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CONTENTS OF VOLUME 56, No. 4, 1954

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PAGE

The use of ion exchangers in the preparation and purification of cytochrome *c*. By E. Margoliash. (With four figures) 529

The chromatographic behaviour of cytochrome *c* on cation exchangers. By E. Margoliash. (With eight figures) 535

Some effects of vitamin B_6 deficiency on fat metabolism in rats. By H. S. R. Desikachar and E. W. McHenry 544

Determination of monoamino monocarboxylic acids by quantitative paper chromatography. By A. R. Kemble and H. T. Macpherson 548

The carotenoids of the carapace of the echinoderm *Ophidiaster ophidianus*. By M. de Nicola. (With two figures) 555

The metabolism of collagen and other proteins of the skin of rabbits. By R. D. Harkness, A. M. Marko, H. M. Muir and A. Neuberger. (With three figures) 558

Content of vitamin A_2 in some Nile fishes. By M. M. Abdullah, S. R. Morcos and M. K. Salah. (With three figures) 569

Studies in immunochemistry. 13. Preparation and properties of the 'O' somatic antigen of *Shigella dysenteriae* (Shiga). By D. A. L. Davies, W. T. J. Morgan and W. Mosimann. (With five figures) . 572

Comparative studies of 'bile salts'. 7. Bile acids of the coypu, *Myocastor coypus*. By G. A. D. Haslewood. (With four figures) 581

On the bromide test of permeability of the barrier between blood and cerebrospinal fluid—an assessment. By G. Hunter, H. V. Smith and L. M. Taylor. (With one figure) 588

The source of antibody globulin in rabbit milk and goat colostrum. By B. A. Askonas, P. N. Campbell, J. H. Humphrey and T. S. Work. (With one figure) 597

Phosphorylation coupled with anaerobic breakdown of citrulline. By V. A. Knivett 602

The effect of arsenate on bacterial citrulline breakdown. By V. A. Knivett. (With two figures) . . 606

Interaction of acids of high molecular weight with albumin. By D. Hamer. (With four figures) . . 610

Phospholipids. 2. Estimation of amino nitrogen in intact phospholipids. By C. H. Lea and D. N. Rhodes. (With three figures) 613

Studies in detoxication. 58. The metabolism of aliphatic alcohols. The glucuronic acid conjugation of chlorinated and some unsaturated alcohols. By J. N. Smith and R. T. Williams 618

A note on the estimation of sphingomyelin in nervous tissue. By R. M. C. Dawson 621

Some enzymic changes in the mammary gland of rats during pregnancy, lactation and mammary involution. By A. L. Greenbaum and F. C. Greenwood. (With six figures) 625

Multiple components in horse-radish peroxidase. By M. A. Jermyn and R. Thomas. (With five figures) . 631

A colorimetric micro-method for the determination of glucose. By B. Mendel, A. Kemp and D. K. Myers. (With six figures) 639

A colorimetric micro-method for the determination of glycogen in tissues. By A. Kemp and A. J. M. Kits van Heijningen 646

Spectrophotometric studies of the reaction of methaemoglobin with hydrogen peroxide. 1. The formation of methaemoglobin-hydrogen peroxide. By K. Dalziel and J. R. P. O'Brien. (With eight figures) . 648

Spectrophotometric studies of the reaction of methaemoglobin with hydrogen peroxide. 2. The degradation of methaemoglobin by hydrogen peroxide. By K. Dalziel and J. R. P. O'Brien. (With seven figures) 660

PAGE

Bile pigment formation *in vitro* from haematin and haem derivatives. By J. E. KENCH. (With six figures) . 669

The measurement of the components of the plasminogen–plasmin system in biological fluids. By A. P. FLETCHER. (With six figures) 677

Derivatives and kinetics of lactoperoxidase. By D. B. MORELL. (With four figures) 683

The isolation and estimation of the steroid oestrogens in placental tissue. By F. L. MITCHELL and R. E. DAVIES. (With six figures) 690

The nature of collagen-chondroitin sulphate linkages in tendon. By D. S. JACKSON. (With three figures) . 699

Chromatography of pyruvic acid and of acetoacetic acid as their 2:4-dinitrophenylhydrazones. By S. MARKEES. (With one figure) 703

Proceedings of the Biochemical Society

The activity of the enzymes sulphatase and glucuronidase in the urine of normal and cancer patients. By E. BOYLAND, D. M. WALLACE and D. C. WILLIAMS xxix

Carbohydrate metabolism and plasma adrenaline level. By H. WEIL-MALHERBE xxix

The quantitative determination of bile pigments in serum using reverse phase partition chromatography. By B. H. BILLING xxx

The specificity of the dinitrobenzene reaction for ketosteroids. By I. E. BROADBENT and W. KLYNE . . xxx

Fluorescent indicators of adsorption in aqueous solution and on the solid phase. By G. WEBER and D. J. R. LAURENCE xxxi

The dye-binding capacity of human plasma determined fluorimetrically, and its relation to the determination of plasma albumin. By J. E. FILDES, D. J. R. LAURENCE and V. H. REES. xxxi

The use of a compound forming fluorescent conjugates with protein in studying the mode of action of polymyxin. By B. A. NEWTON xxxii

Protein-bound polysaccharides in diabetes with and without complications. By I. C. GILLILAND, M. G. HANNO and J. I. STRUDWICK xxxii

Silica and collagen in the lungs and lymph nodes of cortisone-treated silicotic rats. By B. D. STACY and E. J. KING xxxiii

Removal of nucleotide from B_{12} vitamins. By D. E. GANT, E. LESTER SMITH and L. F. J. PARKER . . xxxiv

New purines in B_{12} vitamins. By F. B. BROWN and E. LESTER SMITH xxxiv

Biosynthetic relationships between vitamin B_{12}-like factors. By J. E. FORD and E. S. HOLDSWORTH . xxxv

Observations on the molecular weight of human fibrinogen. By E. A. CASPARY and R. A. KEKWICK . xxxv

Studies on aconitase. By J. F. MORRISON xxxvi

The inhibition of aconitase by fluorocitrate. By J. F. MORRISON and R. A. PETERS xxxvi

pH changes accompanying the formation of metmyoglobin complexes. By P. GEORGE and G. I. H. HANANIA xxxvii

The mechanism of formation of metmyoglobin complexes. By P. GEORGE and G. I. H. HANANIA . xxxviii

'Hyocholic acid', a trihydroxy bile acid from pig bile. By G. A. D. HASLEWOOD xxxviii

Conjugated bile acids of some primate biles. By H. S. WIGGINS xxxix

Infrared spectra of a series of bile acids. By I. D. P. WOOTTON xxxix

Rapid changes in cerebral creatine phosphate. By P. J. HEALD xxxix

A spectrophotometric method for the determination of changes in the thiamine molecule. By J. C. SOMOGYI xl

Citrate accumulation from glycollate by an oxalate decomposing organism. By G. C. N. JAYASURIYA . xli

The isolation of plant nucleic acid fractions for purine and pyrimidine determinations. By H. S. A. SHERRATT and A. J. THOMAS xli

The effect of nucleotoxic stilbene derivatives on globin monolayers. By D. F. CHEESMAN . . . xlii

PAGE

Isolation of cellular constituents using a combination of the Behrens and the Schneider techniques. By G. Adjutantis xlii

A microcuvette for infrared spectrography. By P. Lewis, H. S. Wiggins and I. D. P. Wootton . . xliii

Direct determination of serum cholesterol. By I. MacIntyre and M. Ralston. xliii

The determination of serum calcium with a flame spectrophotometer. By I. MacIntyre xliii

Simplified colorimetric blood sugar method. By A. M. Asatoor and E. J. King xliv

Automatic scanning of paper electrophoretic results. By D. J. R. Laurence xlv

Estimation of salicylates in blood plasma. By E. J. King and V. Pash xlv

Inhibition of enzymes by silicic acid. By P. R. N. Kind, E. J. King, V. Pash, W. Roman and E. Schmidt xlv

Colorimetric estimation of silica without removal of phosphate and iron. By E. J. King, V. Pash and W. Weedon xlvi

Colorimetric estimation of silica in urine. By E. J. King, B. D. Stacy and W. Weedon xlvi

The colorimetric estimation of aluminium. By B. D. Stacy xlvii

The estimation of collagen in silicotic tissues. By B. D. Stacy xlvii

Automatic recording scanner for isotope-bearing chromatograms. By J. E. S. Bradley xlviii

Apparatus for simultaneously measuring ultraviolet adsorption and radioactivity in the eluate from chromatographic columns. By J. E. S. Bradley xlviii

A simplified apparatus for the photo-synthesis of [^{14}C]starch and sugars. By R. W. Dutton . . . xlviii

Apparatus for maintaining and rapidly transferring sliced tissues. By P. J. Heald, H. McIlwain and D. Nelligan xlviii

Paper electrophoresis and chromatography of substances related to vitamin B_{12}. By F. B. Brown, D. E. Gant, E. Lester Smith and L. F. J. Parker xlix

[P. T. O.]

INDEX OF AUTHORS

	PAGE
Abdullah, M. M.	569
Adjutantis, G.	xlii
Asatoor, A. M.	xliv
Askonas, B. A.	597
Billing, B. H.	xxx
Boyland, E.	xxix
Bradley, J. E. S.	xlviii
Broadbent, I. E.	xxx
Brown, F. B.	xxxiv, xlix
Campbell, P. N.	597
Caspary, E. A.	xxxv
Cheesman, D. F.	xlii
Dalziel, K.	648, 660
Davies, D. A. L.	572
Davies, R. E.	690
Dawson, R. M. C.	621
Desikachar, H. S. R.	544
Dutton, R. W.	xlviii
Fildes, J. E.	xxxi
Fletcher, A. P.	677
Ford, J. E.	xxxv
Gant, D. E.	xxxiv, xlix
George, P.	xxxvii, xxxviii
Gilliland, I. C.	xxxii
Greenbaum, A. L.	625
Greenwood, F. C.	625
Hamer, D.	610
Hanania, G. I. H.	xxxvii, xxxviii
Hanno, M. G.	xxxii
Harkness, R. D.	558
Haslewood, G. A. D.	581, xxxviii
Heald, P. J.	xxxix, xlviii
Holdsworth, E. S.	xxxv
Humphrey, J. H.	597
Hunter, G.	588
Jackson, D. S.	699
Jayasuriya, G. C. N.	xli
Jermyn, M. A.	631
Kekwick, R. A.	xxxv
Kemble, A. R.	548
Kemp, A.	639, 646
Kench, J. E.	669
Kind, P. R. N.	xlv
King, E. J.	xxxiii, xliv, xlv, xlvi
Kits van Heijningen, A. J. M.	646
Klyne, W.	xxx
Knivett, V. A.	602, 606
Laurence, D. J. R.	xxxi, xlv
Lea, C. H.	613
Lewis, P.	xliii
McHenry, E. W.	544
McIlwain, H.	xlviii
MacIntyre, I.	xliii
Macpherson, H. T.	548
Margoliash, E.	529, 535
Markees, S.	703
Marko, A. M.	558
Mendel, B.	639
Mitchell, F. L.	690
Morcos, S. R.	569
Morell, D. B.	683
Morgan, W. T. J.	572
Morrison, J. F.	xxxvi
Mosimann, W.	572
Muir, H. M.	558
Myers, D. K.	639
Nelligan, D.	xlviii
Neuberger, A.	558
Newton, B. A.	xxxii
Nicola, M. de	555
O'Brien, J. R. P.	648, 660
Parker, L. F. J.	xxxiv, xlix
Pash, V.	xlv, xlvi
Peters, R. A.	xxxvi
Ralston, M.	xliii
Rees, V. H.	xxxi
Rhodes, D. N.	613
Roman, W.	xlv
Salah, M. K.	569
Schmidt, E.	xlv
Sherratt, H. S. A.	xli
Smith, E. Lester	xxxiv, xlix
Smith, H. V.	588
Smith, J. N.	618
Somogyi, J. C.	xl
Stacy, B. D.	xxxiii, xlvi, xlvii
Strudwick, J. I.	xxxii
Taylor, L. M.	588
Thomas, A. J.	xli
Thomas, R.	631
Wallace, D. M.	xxix
Weber, G.	xxxi
Weedon, W.	xlvi
Weil-Malherbe, H.	xxix
Wiggins, H. S.	xxxix, xliii
Williams, D. C.	xxix
Williams, R. T.	618
Wootton, I. D. P.	xxxix, xliii
Work, T. S.	597

The Use of Ion Exchangers in the Preparation and Purification of Cytochrome *c*

By E. MARGOLIASH*
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(*Received* 16 *July* 1953)

The first mammalian-heart cytochrome *c* preparations (Theorell, 1935; Keilin & Hartree, 1937) had an iron content of 0·34%. However, in 1941 Theorell & Åkeson showed that prolonged electrophoresis of such a preparation in the large-scale Tiselius apparatus resulted in the separation of a relatively colourless component from the bulk of the cytochrome *c*, which now had an iron content of 0·43% (Theorell & Åkeson, 1941).

Since then a number of simpler techniques have been developed for the production of the type of preparation containing 0·43% of iron which are easily applicable to bulk preparations. Keilin & Hartree (1945) used ammonium sulphate fractionation in strongly alkaline solution, and Tsou (1951) precipitation by boiling chloroform. These two techniques are not always reproducible and cause the loss of a variable proportion of the protein by denaturation.

Paléus & Neilands (1950) obtained cytochrome *c* preparations containing 0·42–0·44% iron by adsorption of concentrates on columns of the polycarboxylic cation-exchange resin, Amberlite IRC-50, followed by elution with 0·1 M ammonium acetate/ammonium hydroxide buffer, pH 10·8. However, their main preparation from ox heart was obtained by using as eluent the same buffer at pH 9·0. They recovered three fractions, the purest containing 0·466% iron. The very dilute eluates obtained under these conditions (see Boardman & Partridge, 1953) were concentrated by precipitation with trichloroacetic acid in the cold at pH 3·5 and a low salt concentration. This procedure causes practically complete modification of the cytochrome *c* into an enzymically inactive product spectroscopically identical with native cytochrome *c* (see Margoliash, 1954, Table 2). Neilands (1952) obtained an enzymically active product by eluting the cytochrome *c* from a similar column with a saturated solution of ammonium acetate. The use of such a concentrated solution (over 150 g./100 ml. water) results in considerable dilution during the subsequent dialysis and causes the elution off the column of most of the modified enzymically inactive cytochrome *c* which is normally found in preparations made by using an acid-extraction technique (Margoliash, 1954, and unpublished experiments). He applied the same method to the purification of a concentrate of cytochrome *c* obtained from the rust fungus, *Ustilago sphaerogena*.

The present paper describes a simple and reproducible method for obtaining cytochrome *c* containing 0·43% of iron by the use of cation exchangers, and applicable to large-scale preparations. The same method can be applied to the preparation of reduced and oxidized cytochrome *c* in a stable form. The use of cation exchangers for the direct adsorption of cytochrome *c* from dilute extracts is described, and experiments on the nature of the non-cytochrome fraction of the 0·34% iron type of preparation are reported.

Part of this work was the subject of a preliminary communication (Margoliash, 1952).

MATERIALS AND METHODS

Cation exchangers

Amberlite IRC-50 (Rohm and Haas Co., Philadelphia, U.S.A.). The following adaptation of the various methods used by Paléus & Neilands (1950), Neilands (1952) and Hirs, Moore & Stein (1953) for the preparation of the resin was employed. Using glass-distilled water throughout, 50 g. of the commercially supplied, 50-mesh resin was heated to 60–80° with an excess of 2N-H_2SO_4, washed with water, then heated with an excess of aqueous 2N-NH_3 and was finally washed with water. It was ground wet in a mortar to a smooth paste, stirred up in 300 ml. water, left to settle for exactly 15 sec. and then decanted. The grinding and decantation were repeated until most of the resin had been decanted away. The decanted powder was then again heated with an excess of 2N-NH_3 and the fraction which settled in 1 l. of water between 15 sec. and 5 min. was separated by decantation. The 5 min. settling period was repeated 5 to 6 times in order to remove the finer particles which later would interfere with filtration. The powder was then sucked dry on a sintered glass funnel and left to dry in air until the smell of ammonia was no longer detectable. Yield 15–25 g. Final particle size in the ammonium form, 100–150 mesh.

Decalso F (Permutit Co. Ltd., London) 100-mesh. Using distilled water throughout, this aluminium silicate cation exchanger was prepared in the form of the ammonium salt by washing successively without heating, first with N-H_2SO_4 until an excess of acid remained in the supernatant

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fluid, then with water till the washings were very faintly acid, then with aqueous 0·5 N-NH_3 until the supernatant was alkaline and finally with water till the washings were only very faintly alkaline. The ion exchanger was dried in air.

With both ion exchangers potassium salts were prepared by substituting KOH for the NH_3 used above. The ion exchangers are recovered after use by repeating the alkaline washing of the preparation procedure.

Enzyme preparations and proteins

Heart-muscle preparation was prepared from horse heart according to the method of Keilin & Hartree (1947).

Kidney preparation was prepared according to Slater (1949*b*). The fat-free dry weights of the enzyme preparations were determined according to Slater (1949*b*).

0·34% Iron cytochrome *c* was prepared according to Keilin & Hartree (1945) from horse heart, the final dialysis being overnight against running tap water, followed by three changes of distilled water at 4° to remove salts. Using batches of 8–10 kg. of horse heart the final yield is 12 μmoles of cytochrome *c*/kg. of mince ($\pm$10%).

Crystalline horse-heart myoglobin was prepared by the method of Theorell (1932), and some samples were kindly given by Dr J. C. Kendrew. Globin from this myoglobin was prepared according to Anson & Mirsky's (1930) procedure for globin of haemoglobin. The spectrophotometric data on metmyoglobin and globin were kindly communicated by Dr G. Hanania.

Horse haemoglobin was recrystallized 3 times (Keilin & Hartree, 1935) and globin prepared from it according to Anson & Mirsky (1930).

Commercial bovine serum albumin (Armour and Co.) was used.

Determination of the activity of added cytochrome c *in the succinic-oxidase and cytochrome-oxidase systems*

All measurements were made in Barcroft differential manometers at 37° and pH 7·3 (phosphate buffer). Using the enzyme preparations listed above, the activity of the succinic-oxidase system and of cytochrome oxidase were determined in the test mixtures given by Slater (1949*a*, *b*) using the controls appropriate to each determination. A wide range of added cytochrome *c* concentrations was used (from 1 to 20 μg. of added cytochrome *c* iron per flask), comparing the 0·43% iron preparation obtained with the 0·34% iron type of preparation.

Determination of cytochrome c *concentration*

This was done spectrophotometrically, using a Hilger Uvispec spectrophotometer, by determining the optical density at 550 mμ. of the completely reduced (excess of dithionite) cytochrome *c* solution. The concentration of cytochrome *c* was calculated using a value of 27·7 for the millimolar extinction coefficient ($\epsilon \times 10^{-3}$) for the fully reduced α band at 550 mμ. This value was based on iron determinations of the purest preparations of cytochrome *c* obtained by chromatography on cation exchangers (Margoliash, 1954) and is within the range of values previously reported (Paul, 1951; Paléus & Neilands, 1950). The percentage of reduced cytochrome *c* was calculated from the optical density of the sample at 550 mμ. and the readings when the sample was completely reduced and completely oxidized (excess of $K_3Fe(CN)_6$). This determination was always performed at pH 6·8 (0·1 M phosphate buffer).

Determination of dry weight and iron content. This was done as described by Keilin & Hartree (1945), using the same sample for both determinations, dissolving the iron oxide left after incineration in hot, metal-free N-HCl, and estimating the ferrous dipyridyl from a standard colorimetric curve worked out on a Hilger Uvispec spectrophotometer. This directly determined iron content was never found to be significantly different from that calculated from the optical density at 550 mμ.

Preparation of cytochrome c *containing* 0·43% *iron*

When the object was to obtain a 0·43% iron cytochrome *c* preparation, and not to separate particular chromatographic fractions, nor to obtain the highest possible purification the procedure adopted was as follows:

Preparation with Amberlite IRC-50. The finely divided ammonium salt of the resin (15 g. as prepared above) was added to 100 ml. of an approximately 1% solution of the 0·34% iron cytochrome *c* in water, dialysed as described above. The suspension was gently mixed for 15–30 min. until adsorption was complete. The supernatant non-cytochrome fraction was poured off after the resin had settled completely. Using glass-distilled water, the resin was washed several times by decantation with about 2 l. of water, collected on a coarse, sintered-glass funnel, washed with about 1 l. of water and finally sucked dry.

The cytochrome *c* was then eluted off the resin on the funnel, by suction, with several washings of 10–15 ml. aqueous 0·25 N-NH_3 collecting only the coloured portion of the eluate (Paléus & Neilands, 1950). The eluate was dialysed against running tap water for 4 hr. in a cellophan tube, followed by three to four changes of distilled water. The product was filtered through a Whatman no. 3 filter paper.

The adsorptive capacity of the resin depends on its particle size, and a more concentrated solution of cytochrome *c* is obtained when it is adsorbed on a minimal amount of resin. By careful elution with minimal volumes, the final solution can be obtained 1·5–2 times as concentrated as the original cytochrome *c* preparation. The yield varies from 80 to 95%.

If for particular enzymic studies it was necessary to obtain cytochrome *c* free of ammonium ion, the adsorption was carried out on the potassium salt of the resin, which was eluted with 0·5 M-KCl at neutral pH (Boardman & Partridge, 1953).

Preparation with Decalso F. The procedure was essentially the same as that described for the Amberlite resin, except that 20–25 g. of the ion exchanger were found to be necessary to adsorb all of the cytochrome *c* from 100 ml. of a 1% solution.

It should be noted that the repetition of these simple, non-chromatographic procedures up to 3 times on a sample of cytochrome *c* did not result in a significant increase in iron content above 0·43%.

RESULTS

Table 1 gives the analyses of a number of cytochrome *c* preparations obtained by the simple adsorption and elution methods described.

Fig. 1 shows the comparative activity of cytochrome *c* with 0·34 and 0·43 % iron contents in the succinic-oxidase system using the kidney preparation. Fig. 2 records their comparative activities in the oxidation of ascorbic acid by a normal heart-muscle preparation. It is clear that there are no gross differences of the various enzymic activities per μmole of cytochrome *c* iron. The impurities in the 0·34 % iron preparation had thus no effect on the catalytic activity, and the ion-exchange procedure under the conditions employed had no deleterious effect on the enzymic activity of the cytochrome *c* present in the various preparations.

Table 1. *Purity of cytochrome* c *preparations made by the simple cation-exchanger adsorption technique*

Cation exchanger	Eluting solution	Cytochrome *c* iron (%) Starting material	Product
Decalso F	0·5 M-KCl	0·285	0·423
Decalso F	0·5 M-KCl	0·292	0·418
Decalso F	0·5 M-KCl	0·320	0·427
Decalso F	0·25 M-NH_3	0·335	0·430
Amberlite IRC-50	0·5 M-KCl	0·285	0·427
Amberlite IRC-50	0·5 M-KCl	0·420	0·428
Amberlite IRC-50	0·25 M-NH_3	0·285	0·427
Amberlite IRC-50	0·25 M-NH_3	0·292	0·420
Amberlite IRC-50	0·25 M-NH_3	0·335	0·432

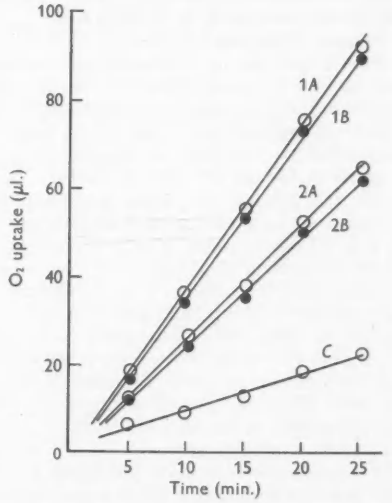

Fig. 1. Comparison of the catalytic effect in the succinic oxidase system of a 0·34 % iron cytochrome *c* (curves *A*, ○) with that of a 0·43 % iron cytochrome *c* (curves *B*, ●) prepared by the simple cation-exchanger adsorption technique. Curves 1*A* and 1*B* represent the O_2 uptake of the system with 2·5 μg. of added cytochrome *c* iron per flask, 2*A* and 2*B* the O_2 uptake with 1·0 μg. of added cytochrome *c* iron per flask, and *C* the O_2 uptake with a control flask containing all the ingredients but no added cytochrome *c*. Each flask contained: 0·1 M phosphate buffer pH 7·3, 0·024 M succinate, 0·78 mg. kidney preparation (fat-free dry weight). Total volume of fluid per flask, 3·3 ml. Temp. 37°.

Preparation of reduced and oxidized cytochrome c *in a stable form*

Paléus & Neilands (1950) first separated chromatographically reduced cytochrome *c* from oxidized cytochrome *c*. The fact that the protein could be eluted off the cation exchanger in either form can be used in the preparation of reduced and oxidized cytochrome *c*.

A solution (100 ml.) containing 80–120 μmoles of horse-heart cytochrome *c* containing 0·43 % of iron, prepared by the cation-exchange method and dialysed against water, is reduced with ascorbic acid or oxidized with $K_3Fe(CN)_6$. (Freshly prepared solutions are used, adding no more than twice the calculated amounts of the reagents. The ascorbic

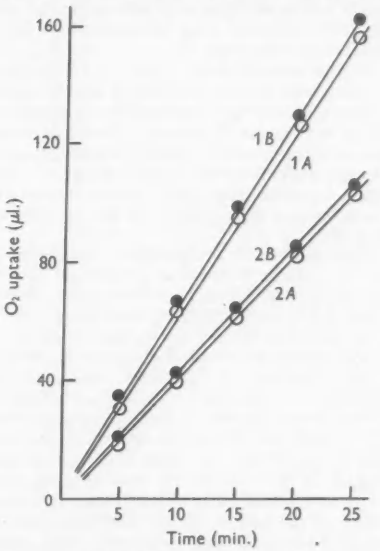

Fig. 2. Comparison of the catalytic effect in the cytochrome oxidase system of a 0·34 % iron cytochrome *c* (curves *A*, ○) with that of a 0·43 % iron cytochrome *c* (curves *B*, ●) prepared by the simple cation-exchanger adsorption technique. Curves 1*A* and 1*B* represent the O_2 uptake of the system with 5·0 μg. of added cytochrome *c* iron per flask, and curves 2*A* and 2*B* the O_2 uptake with 2·5 μg. of added cytochrome *c* iron per flask. Each flask contained: 0·066 M phosphate buffer pH 7·3, 10^{-3} M ethylenediaminetetraacetic acid, 10 mg. ascorbic acid, 0·38 mg. heart-muscle preparation (fat-free dry weight). Total volume of fluid per flask, 3·3 ml. Temp. 37°. The O_2 uptake values plotted were obtained by deduction of the O_2 uptake of control (containing at each concentration of added cytochrome *c*, all the ingredients except the cytochrome oxidase) from that of the complete system.

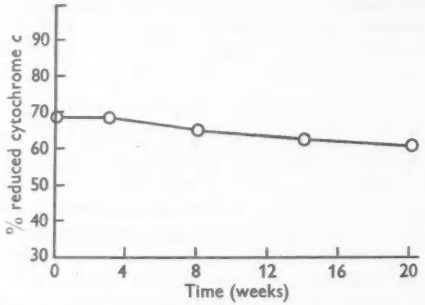

Fig. 3. Storage of freeze-dried reduced cytochrome *c*. Freeze-dried cytochrome c powdered, sealed in ampoules under N_2 and kept at 0–4°.

acid is neutralized with 5% (w/v) NH_3 and ethylenediaminetetraacetic acid is added to stop auto-oxidation.) Alternatively, the cytochrome *c* can be reduced by a minimal amount of sodium dithionite.

The solution is gently shaken with 18 g. of the potassium or ammonium salt of Amberlite IRC-50 per 100 μmoles of cytochrome *c*. The mixing is continued for 15–30 min. until adsorption is complete. The resin is collected on a coarse, sintered-glass funnel, and if the filtrate contains an appreciable amount of cytochrome *c*, a further adsorption is carried out using 3–5 g. of the resin. All the cation exchanger is then washed on the filter using about 2 l. of glass-distilled water and sucked dry.

Oxidized cytochrome *c* can be eluted from the resin with four to six washings of 20 ml. of 0·25 M-NH_3. It is then filtered through a Whatman no. 3 filter paper and freeze-dried over H_2SO_4 and NaOH. The yield is 90–97%.

For the elution of reduced cytochrome *c* from the resin both strongly alkaline solutions and high salt concentrations must be avoided since both greatly increase the auto-oxidizability of cytochrome *c*. The elution is performed either with 0·25 M aqueous ammonia (final pH of eluate, 9–10), or with 0·25 M-KCl at neutrality. In both cases it is essential to dialyse away the alkali or the salt as rapidly as possible in the cold (0–4°), with shaking, against 5 changes of distilled water within 4–5 hr. The material remaining in the bag is filtered and freeze-dried over H_2SO_4 and NaOH. The yield is 90–95% using ammonia elution, and is usually somewhat lower with KCl. The cytochrome *c* is 70–85% reduced. The products are powdered and kept in sealed ampoules under nitrogen. Such powders stored at 0° for 6 months give clear solutions in water, have substantially the same percentage of reduced or of oxidized cytochrome *c* as when prepared (Fig. 3) and are fully active in the succinic oxidase and cytochrome oxidase systems.

The use of cation exchangers for the concentration of extracts containing cytochrome c

The marked ability of cation exchangers to adsorb cytochrome *c* from solution (Paléus & Neilands, 1950) gives an easy method for concentrating extracts having a low cytochrome *c* content. This permits the preparation of the protein from sources which would otherwise entail considerable difficulties of concentration. In any such procedure the following points are important: (1) Efficient adsorption of cytochrome *c* at pH 7 on to Amberlite IRC-50 occurs readily at total cation concentrations below 0·03 g. ion/l. At much lower cation concentrations the adsorption becomes too efficient and the entire cytochrome *c* accumulates in a thin sharp band causing partial clogging of the column. (2) For maximal purification of the adsorbed cytochrome *c* it is essential to develop the chromatogram as slowly as practicable. If the adsorbed protein is washed with water only, then eluted rapidly, there will be no further purification after that obtained at the initial absorption. Thus simple, non-chromatographic procedures, such as described under Methods, give products containing 0·43% iron, while chromatographic procedures with slow elutions, such as employed by Paléus & Neilands (1950), give preparations having up to 0·466% iron. The procedure generally found suitable for the concentration of dilute extracts containing cytochrome *c* was essentially that employed by Neilands (1952) for the purification of concentrates.

A column of the ammonium salt of Amberlite IRC-50 as prepared above, of dimensions 12 × 3 cm., is adequate for the adsorption of about 50 μmoles of cytochrome *c*. The extract is neutralized to pH 7 and dialysed against running tap water till the salt concentration is reduced to 0·01–0·03 M. Any precipitate is filtered off and the extract passed through the column at about 4 ml./min. The adsorbed protein is washed with water (500 ml.) and then with 0·1 M ammonium acetate (from 2 to 7 l.) until the cytochrome band has spread over one-half to two-thirds of the column. The cytochrome *c* is finally eluted in a sharp band with aqueous 0·25 M ammonia at a rate not exceeding 0·2–0·3 ml./min. Table 2 gives the results of analyses of several preparations from a single batch of minced horse heart made with different methods of extraction, using direct adsorption on an Amberlite IRC-50 column, and different washing solutions.

The considerable variation in the degree of purity of the products emphasizes the fact that although an adsorption procedure with a cation exchanger leads to excellent purification when the starting material is a Keilin & Hartree preparation containing 0·34% iron, this is not necessarily the case with any material containing cytochrome *c*. The purity of the product depends on the acidity and the concentration of the impurities present in the chromatographed solution. It is notable that the percentage of reduced cytochrome *c* in all these preparations is much higher than in the usual Keilin & Hartree preparation. This is because cytochrome *c* in the first extract is always largely

Table 2. *Analysis of cytochrome* c *preparations made by direct adsorption of horse-heart mince extracts on Amberlite IRC-50*

TCA represents trichloroacetic acid; NH_4Ac represents ammonium acetate.

	Extraction				Product		
Horse-heart mince	Solution	Time (hr.)	pH	Washed on column with	Fe (%)	Reduced (%)	$E_{550\,m\mu}/E_{280\,m\mu}$
Unwashed	2·5% TCA	3	4·5	Water	0·242	66	0·907
Unwashed	2·5% TCA	15	4·5	Water	0·321	85	1·010
Washed with water	1·5% TCA	3	4·5	Water	0·282	74	0·940
Washed with water	0·5 M-NH_4Ac	3	7·0	Water	0·338	50	1·005
Unwashed	2·5% TCA	15	4·5	0·05 M-NH_4.Ac	0·386	71	1·100
Unwashed	2·5% TCA	3	4·5	0·1 M-NH_4.Ac	0·440	50	1·180

Table 3. *Reconstitution of the 0·34% iron type of cytochrome* c *preparation. Starting preparation contained 0·43% iron*

Added substance	Product cytochrome Fe (%)
None	0·418
Denatured globin from horse haemoglobin	0·425
Horse haemoglobin	0·412
Denatured globin from horse-heart myoglobin	0·419
Native globin from horse-heart myoglobin	0·330
Horse-heart myoglobin	0·325
Bovine serum albumin	0·402
Non-cytochrome fraction of 0·34% iron preparation	0·313

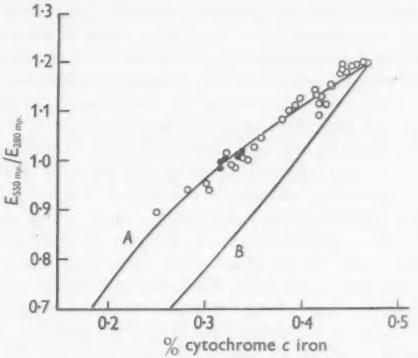

Fig. 4. The relation of the ratio of the optical densities (E) at 550 mμ. (reduced cytochrome *c*) and 280 mμ. (oxidized cytochrome *c*) to the percentage of cytochrome *c* iron, for various preparations of horse-heart cytochrome *c*. Points (○) refer to preparations which had at some stage been adsorbed on to a cation exchanger, while points (●) refer to preparations made by the method of Keilin & Hartree (1945). The percentage of cytochrome *c* iron for each preparation was calculated from the optical density at 550 mμ. (reduced cytochrome *c*) and the dry weight, as given under Methods. Curve *A* was constructed assuming that the sole non-cytochrome impurity was horse-heart myoglobin globin, while curve *B* was constructed assuming the sole impurity was acid metmyoglobin. The millimolar extinction coefficients used were: at 280 mμ. for globin from myoglobin, 13·1; for acid metmyoglobin, 32·1; for pure ferricytochrome *c*, 23·0: at 550 mμ. for pure ferrocytochrome *c*, 27·7.

reduced by the denatured protein in the extract, and it is not treated with acid as in the Keilin & Hartree procedure.

The nature of the non-cytochrome component in the 0·34% iron cytochrome c *preparation*

When a horse-heart cytochrome *c* preparation containing 0·34% iron was adsorbed on a cation exchanger at neutral pH the supernatant liquid was yellow to brown. Visual spectroscopy showed the presence of a little myoglobin, and a very small amount of modified cytochrome *c*, showing a rather hazy α band, but not reacting to any large extent with carbon monoxide. The total protein as determined by the dry weight of the ethanol-washed trichloroacetic acid precipitate was always considerably in excess of that accounted for by the spectroscopically visible constituents.

Reconstitution of cytochrome c *containing 0·34% iron.* Reconstitution of the 0·34% iron preparation was obtained as follows. A 0·43% iron cytochrome *c* preparation was divided into samples of 30 μmoles of cytochrome *c* and each sample diluted to 250 ml. with water. To each sample 200 mg. of one of the various proteins listed in Table 3 were added, the pH was adjusted to 4·5 with N trichloroacetic acid and the mixture left at room temperature for 4 hr. This is the pH at which the extraction with dilute trichloroacetic acid is carried out in the Keilin & Hartree (1945) procedure for the preparation of the 0·34% iron cytochrome *c*. The final steps of this procedure, starting with the neutralization of the extract and the first addition of ammonium sulphate, were followed, and the products analysed for iron. Table 3 shows that reconstitution occurred only with myoglobin, native globin from myoglobin and the non-cytochrome fraction of the 0·34% iron preparation.

Ultraviolet absorption. Fig. 4 is a plot of the ratio of the optical densities at 280 mμ. (completely oxidized) and at 550 mμ. (after reduction)

against percentage iron in the preparation, for a number of horse-heart cytochrome *c* preparations ranging from 0·2 to 0·45 % iron. These included preparations made using cation exchangers and also by the usual method. The empirical curve in Fig. 4 can be used to estimate the purity of any preparation of horse-heart cytochrome *c* from simple spectrophotometric determinations. This obviates the otherwise necessary iron and dry-weight estimations. The solid line *A* in Fig. 4 was constructed assuming the impurity to be the globin of myoglobin. The good agreement of this line with the experimental points is consistent with the hypothesis that the non-cytochrome fraction in the 0·34 % iron preparation was this globin. Line *B* was constructed assuming the impurity to be acid metmyoglobin, and neglecting its absorption at 550 mμ.

Paper electrophoresis of the non-cytochrome fraction of the preparations containing 0·34 % iron. The non-cytochrome fractions of a number of 0·34 % iron cytochrome *c* preparations were isolated by the cation-exchanger technique, treated twice with fresh Amberlite IRC-50 in order to remove all traces of cytochrome *c*, and concentrated. Each was then subjected to electrophoresis on paper using the method of Markham & Smith (1952). Controls were always run simultaneously on the same strip of paper, and each comparison was carried out at at least three different pH values between 4·5 and 9·5. The non-cytochrome fraction showed a single component with a mobility identical with that of native globin prepared from horse-heart myoglobin, at each pH tested. It was found essential to carry out the concentration of the non-cytochrome fraction very carefully. If during freeze drying a small part of the ice melted, if the solution were frozen and thawed several times, or dried from the liquid state, there appeared a second electrophoretic band of modified globin, with an isoelectric point below that of native globin (between pH 5 and 6). Similar artifacts were obtained with native globin prepared from pure myoglobin. Freeze drying at temperatures well below the freezing point of the solution was found to be most satisfactory.

In conclusion, the evidence presented above indicates that the non-cytochrome fraction of the Keilin & Hartree 0·34 % iron preparation consists of globin from myoglobin, which is present in the trichloroacetic acid extract of heart mince. The very small amount of myoglobin accounts for the colour of the non-cytochrome fraction.

DISCUSSION

The methods described above give cytochrome *c* preparations having a 0·43 % iron content, the product being fully enzymically active. This product is probably a mixture of the various cytochrome *c* fractions which Paléus & Neilands (1950) obtained by chromatography on Amberlite IRC-50. The method is essentially a simplification of their technique, but does not overcome the disadvantage of the method of Neilands (1952) since the product includes that fraction of enzymically inactive modified cytochrome *c* which is normally found in concentrates prepared by acid extraction (Margoliash, 1954). The preparation of 0·43 % iron cytochrome *c* can however be carried out without the use of a column and the product is obtained directly in concentrated solution.

The advantage of this method over others (Theorell & Åkeson, 1941; Keilin & Hartree, 1945; Tsou, 1951) is its simplicity and reliability together with a practically quantitative yield. There is no great difficulty in obtaining concentrated solutions of oxidized cytochrome *c*, since the usual preparations are largely oxidized. Concentrated solutions of reduced cytochrome *c* have, on the other hand, always been difficult to obtain. The method usually applied is to bubble hydrogen gas through the solution in the presence of platinum black. This technique is successful with rather dilute solutions of cytochrome *c* but with more concentrated solutions the process is tedious and the degree of reduction obtained is often too low to be useful in enzymic studies. Other methods involve the use of reducing agents, usually ascorbic acid, which leave reaction products in the solution, and these may interfere with the enzymic reaction studied. In the technique described in this paper, the observation that cytochrome *c* can be adsorbed and eluted from a cation exchanger while remaining largely oxidized or reduced was used. The reaction products of the reducing or oxidizing agents were washed away before eluting the cytochrome and thus concentrated solutions of reduced or oxidized cytochrome *c* were obtained and could be freeze-dried to stable powders.

The practical advantages of the method given above over the techniques which have been hitherto used are the stability of the dried product in the reduced or oxidized form, the possibility of obtaining solutions of any desired strength, and the complete removal of the chemical agents used for reduction or oxidation and their reaction products. The main application of such preparations will probably be to enzymic studies requiring the use of stoicheiometric rather than catalytic amounts of cytochrome *c*.

The use of cation exchangers has made possible the separation of the non-cytochrome fraction of the 0·34 % iron type of preparation, free from any cytochrome *c*. In the horse-heart preparation it consists of globin from myoglobin. The amount of myoglobin in the non-cytochrome fraction depends

on whether globin is dissociated from the haem at the pH of the original extraction. Thus in the usual Keilin & Hartree preparation of cytochrome *c* (pH of extraction 4·5) there are no more than traces of myoglobin, whereas if the extraction of the heart mince is carried out at neutral pH, as with 0·5M ammonium acetate, most of the non-cytochrome fraction consists of myoglobin.

SUMMARY

1. A simple and reliable procedure using cation exchangers to prepare 0·43% iron cytochrome *c* from the 0·34% iron preparation from horse heart is described. The product is as active enzymically (per μmole of cytochrome *c* iron) as the preparation containing 0·34% iron.

2. An application of this technique is made to the preparation of cytochrome *c* in either the reduced or oxidized state, in a stable form. This enables the preparation of solutions of reduced or oxidized cytochrome *c* in any desired concentration, whenever required and entirely free from reducing or oxidizing agents and their reaction products. After storage the dried products are enzymically fully active.

3. The non-cytochrome fraction of the 0·34% iron cytochrome *c* preparations is identified as globin from myoglobin. Under suitable conditions cytochrome *c* can react with myoglobin or the globin prepared from it.

The author wishes to express his gratitude to Prof. D. Keilin, F.R.S., for his constant advice, encouragement and criticism during this work. He is indebted to Dr E. C. Slater for reading the paper, and to Dr R. Markham for instruction in paper electrophoresis. He also thanks Mr N. K. Boardman and Dr S. M. Partridge for their invaluable help in the use of ion exchangers.

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The Chromatographic Behaviour of Cytochrome *c* on Cation Exchangers

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Paléus & Neilands (1950) first chromatographed an ox-heart cytochrome *c* preparation containing 0·34% of iron on Amberlite IRC-50 (a synthetic, polycarboxylic cation exchanger). They obtained three fractions: the first was reduced cytochrome *c*, containing 0·401% iron, the second oxidized cytochrome *c*, containing 0·466% iron, while the third contained 0·353% iron.

Boardman & Partridge (1953) studied quantitatively the effect of the cation concentration and pH on the chromatography of cytochrome *c* on Amberlite IRC-50. The cytochrome *c* could not be eluted below pH 6, the elution volume decreased rapidly from pH 6 to 7, remained stationary from pH 7 to 8, then decreased again to pH 10. This behaviour was explained by the summation of two forces affecting the protein—resin interaction, short-range forces which bind the cytochrome *c* irreversibly below pH 6, and the normal, electrostatic attraction depending both on the ionization of the resin, which is depressed as the pH decreases, and the ionization of the protein, which is depressed as the pH increases. At pH 7 cytochrome *c* could be eluted using univalent cations at concentrations above 0·22 g. ions/l.

In the course of work on the use of cation exchangers for the concentration and further purification of cytochrome *c* (Margoliash, 1952, 1954), it

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was found necessary to study the chromatographic behaviour of the protein, as well as the catalytic properties of the various fractions separated. This paper presents the results of this work.

MATERIALS AND METHODS

Amberlite IRC-50 (Rohm and Haas Co., Philadelphia, U.S.A.) as the ammonium salt, the heart-muscle preparation, the kidney preparation and the 0·34% iron cytochrome *c* were prepared as described in the previous paper (Margoliash, 1954).

The activity of cytochrome *c* fractions in the succinic-oxidase and cytochrome-oxidase systems, the total cytochrome *c* concentration and the percentage of reduced cytochrome *c* as well as the dry weight and the iron content of the various fractions were determined as given in the previous paper (Margoliash, 1954).

Ascorbic acid oxidation by cytochrome *c* preparations was measured in the test mixture used in the determination of the cytochrome *c* activity in the cytochrome-oxidase system, with ascorbic acid, but in the absence of the cytochrome-oxidase preparation. The pH was 7·3, and the final concentration of phosphate buffer was 0·066 M. All flasks contained 1×10^{-3} M ethylenediaminetetraacetic acid. A simultaneous blank determination was always carried out with ascorbic acid in the absence of cytochrome *c*, and the resulting measure of the rate of auto-oxidation of the ascorbic acid was subtracted from the values obtained with cytochrome *c*, to give the rate of oxidation of ascorbic acid by the cytochrome *c* preparation.

It should be emphasized that this is not equivalent to a measure of the rate of auto-oxidation of the cytochrome *c* but represents an oxidation of ascorbic acid catalysed by a small amount of an impurity in the preparation (see below, also Tsou, 1951).

RESULTS

Chromatography at pH 7

The effect of the cation concentration and the pH of the eluting solution on the movement of horse-heart cytochrome *c* adsorbed on a column of Amberlite IRC-50 was found to be the same as that described by Boardman & Partridge (1953). The elution fluids usually used in these experiments were: ammonium acetate + acetic acid buffers below pH 7, ammonium acetate at neutrality and ammonium acetate + ammonia buffers, as used by Paléus & Neilands (1950) above pH 7. Similar results were obtained using solutions of Na^+ or K^+ salts, with the appropriate salt of the resin (Boardman & Partridge, 1953).

Using 0·25 M ammonium acetate as elution fluid, chromatography of a 0·34% iron cytochrome *c* preparation, at pH 7, gave three fractions. First, a forerunning, non-cytochrome fraction which came off very soon after the beginning of elution, probably at the fluid front. This fraction corresponded to the non-adsorbed fraction when a 0·34% iron preparation is adsorbed on the resin (see previous paper, Margoliash, 1954). Secondly, *Fraction I* pH 7, a cytochrome fraction which contained from 75 to 90% of the total amount of cytochrome *c* put on the column, and came off in a fairly wide band. Thirdly, *Fraction II* pH 7, which was the remaining 10–25% of the cytochrome *c* which did not appear to move at all with the elution fluid containing 0·25 g. ions/l., and which was eluted as a single band with 0·5 N ammonium hydroxide.

Fig. 1 records a typical chromatogram at pH 7.

Chromatography at pH 9·6

When fraction I pH 7 was dialysed against distilled water until salt free and was rechromatographed at pH 9·6 (0·25 M buffer) it separated into three fractions. First, a very small, forerunning, non-cytochrome fraction which showed under the spectroscope the same compounds as the previous non-cytochrome fraction, except that there was no modified cytochrome *c*. It came off very soon after the beginning of elution like the non-cytochrome fraction obtained at pH 7. Secondly, *Fraction I* pH 9·6, which was a very small, distinct, fast-running cytochrome *c* band which constituted about 1–3% of the total cytochrome *c*. Thirdly, *Fraction II* pH 9·6, which contained the rest of the cytochrome put on the column. When this fraction was made to move slowly down the column it separated into a forerunning reduced fraction followed by the rest of the fraction in the oxidized form, as described by Paléus & Neilands (1950).

Fig. 2 records a typical chromatogram at pH 9·6, in which fraction II was eluted rapidly, so that there was no distinct separation of the reduced and oxidized parts.

Elution of reduced and oxidized cytochrome c

The separation of reduced and oxidized cytochrome *c* on columns of Amberlite IRC-50 was first achieved by Paléus & Neilands (1950). In all our experiments reduced cytochrome *c* separated as a distinct chromatographic fraction if the rate of elution were slow enough. Comparison of the elution curves for reduced and oxidized cytochrome *c* (Figs. 1, 2) shows that with every fraction eluted below the pH at which cytochrome *c* becomes readily auto-oxidizable (about pH 11) there was a peak of reduced cytochrome *c* slightly preceding the peak of the oxidized form. Fig. 3 shows the elution diagram of the total fraction II pH 9·6, containing both ferro- and ferri-cytochrome *c*, when rechromatographed at pH 7 at about one-third the rate used in the two previous runs. The reduced and oxidized forms have nearly separated, producing distinct peaks.

This separation is not a property peculiar to cation-exchange columns, as similar effects were noted on columns of calcium phosphate gel; indeed,

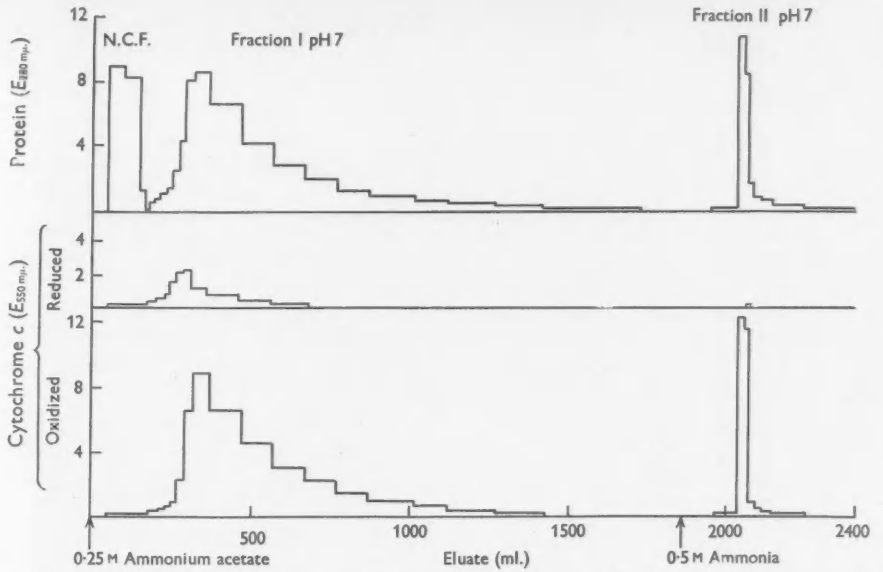

Fig. 1. Elution chromatogram of a 0·34% iron cytochrome *c* preparation at pH 7. N.C.F.: non-cytochrome fraction. Column of Amberlite IRC-50; ammonium salt, 35 × 3 cm. Rate of flow: 1·3–1·5 ml./min. Total of 142 μmoles of cytochrome *c* adsorbed on the column. Elution with 0·25 M ammonium acetate, then 0·5 N-NH_3. Ordinates: optical densities of eluate at 280 mμ. and at 550 mμ. for ferrocytochrome *c*, and for ferricytochrome *c* after reduction.

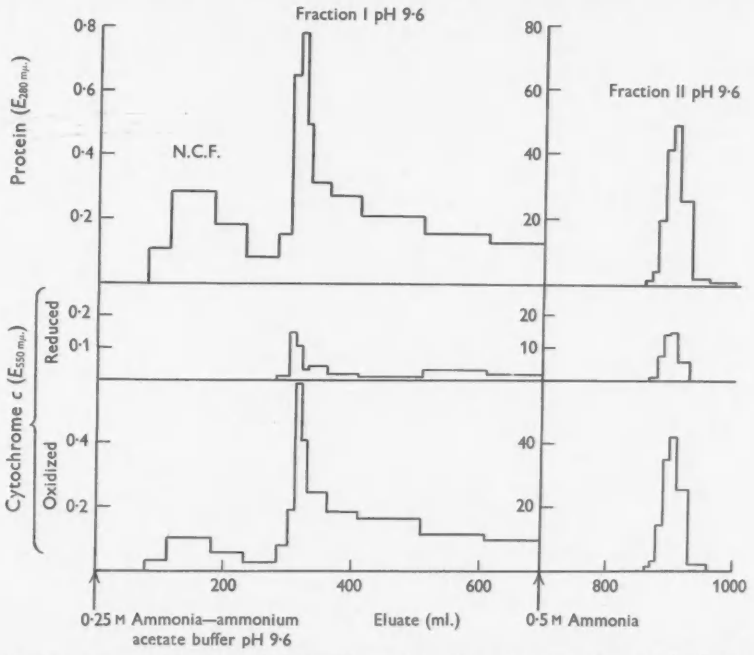

Fig. 2. Elution chromatogram of fraction I pH 7 at pH 9·6. Total of 75 μmoles of cytochrome *c* adsorbed on the column. Elution with 0·25 M ammonium acetate + ammonium hydroxide buffer pH 9·6, then with 0·5 N-NH_3. Column, rate of flow, ordinates and abbreviations as in Fig. 1.

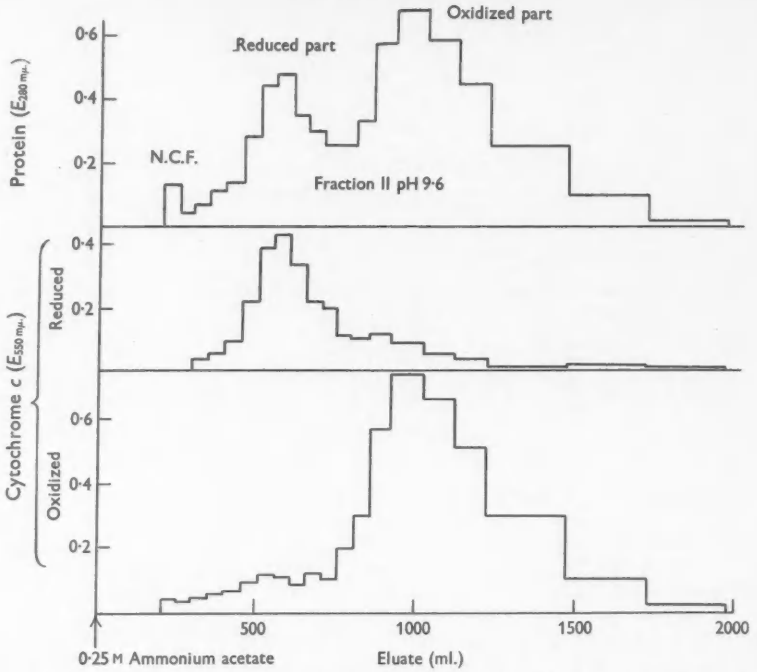

Fig. 3. Elution chromatogram of fraction II pH 9·6, at pH 7. Total of 20 μmoles of cytochrome *c* adsorbed on the column. Elution with 0·25 M ammonium acetate. Rate of flow 0·2–0·3 ml./min. Column, ordinates and abbreviations as in Fig. 1.

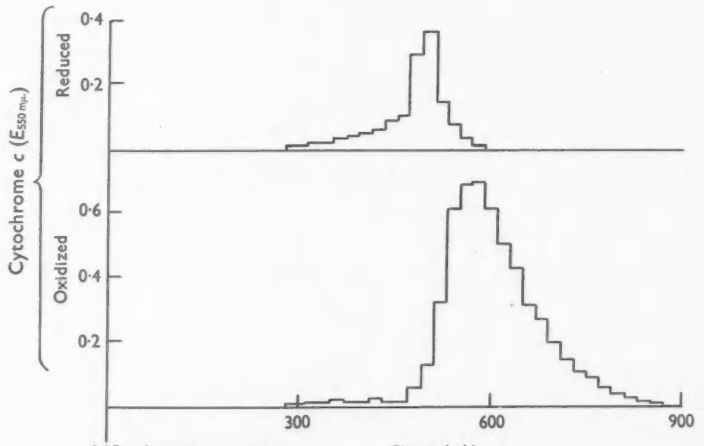

Fig. 4. Elution chromatogram at pH 7 of a fraction II pH 9·6 (oxidized), which had been artificially reduced to the extent of 21·5%. Column of Amberlite IRC-50, ammonium salt, 25 × 1 cm. Rate of flow 0·5 ml./min. Total of 4·9 μmoles of cytochrome *c* adsorbed on column. Elution with 0·25 M ammonium acetate. Ordinates: optical densities at 550 mμ. for ferrocytochrome *c*, and at 550 mμ. for ferricytochrome *c* after reduction.

the tendency of cytochrome *c* to precipitate when oxidized, and redissolve when reduced was used by Keilin (1930) in the preparation of yeast cytochrome *c*, while Zeile & Reuter (1933) showed that oxidized cytochrome *c* is adsorbed on kaolin, while under the same conditions the reduced form is not.

In order to test whether the reduced and oxidized parts of a fraction separated on the chromatogram entirely by virtue of their state of oxidation, the following experiment was performed. The starting material was the oxidized part of fraction II pH 9·6, which had been separated from the reduced part at pH 7 in a run similar to that recorded in Fig. 3, and chromatographed a second time at pH 7 to remove nearly all traces of the reduced material. It was then 99·6 % oxidized. A portion of this material was prepared in the reduced form by the cation-exchange procedure using sodium dithionite (Margoliash, 1954) and mixed with the original material to give a 21·5 % reduced fraction II pH 9·6. This was chromatographed at pH 7, and Fig. 4 records the elution diagram. It shows that although the reduced and oxidized cytochromes were originally the same chromatographic species, they separated when eluted slowly, the reduced fraction in the eluate being 19·1 % of the total.

Reduction of cytochrome c *during chromatography*

The original preparation used in the experiments reported in Figs. 1–3 was 3 % reduced; it is possible to calculate from the elution diagrams that fraction I pH 7 represented 89 % of the total cytochrome *c* put on the column at pH 7 and was 11·7 % reduced, while fraction II pH 9·6 represented 97 % of the total cytochrome *c* put on the column at pH 9·6 and was 24·9 % reduced. When the total fraction II pH 9·6 was rechromatographed at pH 7, so as to separate ferro- and ferri-cytochrome *c*, all the cytochrome *c* put on the column was recovered, 25·9 % in the reduced fraction, and 74·1 % in the oxidized fraction.

Thus, cytochrome *c* is partly reduced on a resin column, the degree of reduction increasing with the pH up to pH 11, where the auto-oxidation of cytochrome *c* becomes very rapid. When partial reduction had been caused by chromatography at a high pH, a second chromatography at a lower pH did not result in reoxidation of any of the reduced cytochrome *c* although the proportion of the latter was grossly above that which was normally reduced at the lower pH. In solution, however, reduction of cytochrome *c* did not take place even at pH 10. Reduction only occurred when cytochrome *c* was adsorbed on to some solid. This was not a property unique to the cation exchanger used, as the same phenomenon was readily observed on other cation exchangers such as Decalso F (Permutit Co. Ltd., London), and also on other adsorbents such as a calcium phosphate gel column.

Rate of ascorbic acid oxidation by the cytochrome c *fractions*

Table 1 records the rates of ascorbic acid oxidation by the original 0·34 % iron cytochrome *c* preparation used, and by the various fractions separated from it by chromatography at pH 7 and pH 9·6 on Amberlite IRC-50. There was a decrease of the rate of ascorbic acid oxidation as the cytochrome fractions were further chromatographed. This is consonant with the hypothesis that the oxidation of ascorbic acid by cytochrome *c* preparations is due to an impurity or to a modified form of cytochrome *c*, which is gradually removed by chromatography.

Table 1. *Activity in the cytochrome-oxidase system, rate of ascorbic acid oxidation and analysis of various chromatographic fractions of cytochrome* c

Cytochrome *c* fraction	Activity in cytochrome-oxidase system* (μl. O_2/hr./flask)	Rate of ascorbic acid oxidation* (μl. O_2/hr./flask)	Percentage of total preparation	Iron (%)	Reduced (%)	$\frac{E_{550\ m\mu.\ (reduced)}}{E_{280\ m\mu.\ (oxidized)}}$
Keilin & Hartree preparation	376	68	100	0·334	3	1·007
Fraction I pH 7	390	40	89	0·418	11·7	1·115
Fraction II pH 7	142	123	11	0·377	0·5	1·085
Fraction I pH 9·6	354	112	3	0·344	9·5	1·000
Fraction II pH 9·6 (total)	357	31	86	0·432	24·9	1·152
Fraction II pH 9·6 (reduced part)	351	42	22	0·413	65·8	1·120
Fraction II pH 9·6 (oxidized part)	367	19	64	0·452	5·3	1·185

* Both rates of O_2 uptake were measured in 0·066 M phosphate buffer pH 7·3, 1×10^{-3} M ethylenediaminetetraacetic acid, $2{\cdot}72 \times 10^{-5}$ M added cytochrome *c* at 37°. 0·372 mg. Heart-muscle preparation (fat-free dry weight) was added to the flasks measuring cytochrome-oxidase activity. The O_2 uptakes of the flasks measuring ascorbic acid oxidation, containing all the ingredients except the oxidase, were used as the corresponding blank values.

Table 2. *Percentage of total cytochrome* c *separated as fraction II* pH 7 *from various preparations*

TCA represents trichloroacetic acid; NH_4Ac represents ammonium acetate.

Starting material	Extraction conditions: Solution used	pH	Time (hr.)	Amount of fraction II pH 7 as % of total cytochrome *c*
Horse-heart mince	2·5% TCA	4·5	3	11
Horse-heart mince	2·5% TCA	4·5	15	19
Washed horse-heart mince	1·5% TCA	4·5	3	0·7
Washed horse-heart mince	0·5M-NH_4Ac	7·0	3	0
Fraction I pH 7	2·5% TCA	4·5	3	56

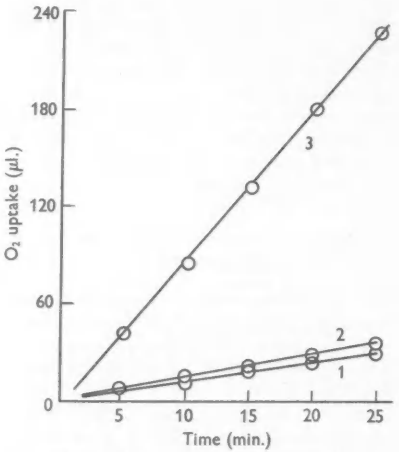

Fig. 5. Catalytic effects in the cytochrome-oxidase system of a normal 0·34% iron cytochrome *c* preparation (3), a fraction II pH 7 artificially prepared from a fraction I pH 7 by incubation with trichloroacetic acid (2), and a fraction II pH 7 separated from the original 0·34% iron preparation (1). Each flask contained: 0·066M phosphate buffer pH 7·3, 10^{-3}M ethylenediaminetetraacetic acid, 10 mg. ascorbic acid, 0·252 mg. heart-muscle preparation (fat-free dry wt.), 10 μg. of cytochrome *c* iron. Total volume: 3·3 ml. Temperature 37°. The values plotted were obtained by deduction of the O_2 uptake of the controls containing all the ingredients except the cytochrome oxidase, from that of the complete system.

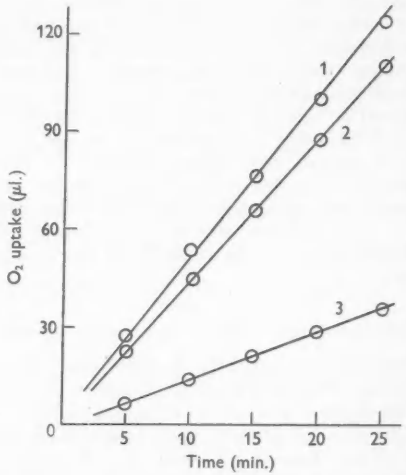

Fig. 6. Rates of ascorbic acid oxidation by a normal 0·34% iron cytochrome *c* preparation (3), a fraction II pH 7 artificially prepared from a fraction I pH 7 by incubation with trichloroacetic acid (2), and a fraction II pH 7 separated from the original 0·34% iron preparation (1). Each flask contained: 0·066M phosphate buffer pH 7·3, 10^{-3}M ethylenediaminetetraacetic acid, 10 mg. ascorbic acid, 10 μg. of cytochrome *c* iron. Total volume of fluid, 3·3 ml. Temperature 37°. The values plotted were obtained by deduction of the O_2 uptake of a control containing all the ingredients except cytochrome *c*, from that of the complete system.

Fraction II pH 7 showed an unusually high rate of ascorbic acid oxidation and low activity in the cytochrome-oxidase system. The properties of this fraction placed it in a class of its own, and it is discussed separately.

Enzymic activity of the various cytochrome c *fractions*

Table 1 records the activity of some of the various cytochrome *c* fractions in the cytochrome-oxidase system, using heart-muscle preparation, at a concentration of 5 μg. of added cytochrome *c* iron per flask. These fractions had similar activities and calculated on the basis of cytochrome *c* iron but not on total protein, these activities were the same as that of the original 0·34% iron preparation, which contained about 27% non-cytochrome protein. The only exception to this generalization is fraction II pH 7. A similar study of the activity of the various fractions in the succinic-oxidase system of kidney preparation gave identical results.

The nature of fraction II pH 7

Fraction II pH 7 differed considerably from all the other fractions and from the original cytochrome *c* preparation in having a high rate of ascorbic acid oxidation and low enzymic activity. Visual spectroscopy, however, did not show any difference between this fraction and the others. It did not appear to react appreciably with carbon monoxide and there was no intensification of the spectrum after treating with pyridine and dithionite. The question arose as to whether this unusual cytochrome *c* fraction was an artifact of the preparation, or was preformed in the tissue. In order to decide between these alternatives the following experiments were performed.

Table 3. *Data on the purest cytochrome* c *fraction obtained by chromatography on Amberlite IRC-50*

Iron (%)		0·465
$\epsilon_{550\,m\mu.} \times 10^{-4}$	Reduced	2·77
	Oxidized	0·92
$\frac{E_{550\,m\mu.\,(reduced)}}{E_{280\,m\mu.\,(oxidized)}}$		1·192
Rate of ascorbic acid oxidation* (μl. O_2/hr./flask)		12
Equivalent weight†		12 000

* Determined as in Table 1.
† Calculated from the iron determinations.

Fresh horse-heart mince was divided into 1 kg. lots and extracted by different methods. The fluid was pressed out in a hydraulic press. The extracts were neutralized to pH 7·3, dialysed overnight against running tap water, filtered and adsorbed directly on to a column of Amberlite IRC-50. After being washed, the adsorbed cytochrome *c* was eluted with 0·25 M ammonium acetate at pH 7 (which removed only fraction I pH 7) and then with 0·5 M-NH_3 (which eluted fraction II pH 7). The percentage of the total present as fraction II pH 7 was calculated from spectrophotometric determinations. Simultaneously, 30 μmoles of a pure fraction I pH 7 which had been freed completely from fraction II pH 7, by chromatographing twice at pH 7, was kept at room temperature for 3 hr. in 2·5% trichloroacetic acid brought to pH 4·5 with 2 N-NH_3. This caused the cytochrome *c* to be precipitated. It was then redissolved by neutralization and treated as the extracts above.

The results presented in Table 2 show that fraction II pH 7 appeared in extracts containing cytochrome *c* only when acid was used for extraction. The more concentrated the acid and the longer the time of extraction, the more of fraction II pH 7 appeared. Moreover, trichloroacetic acid at the

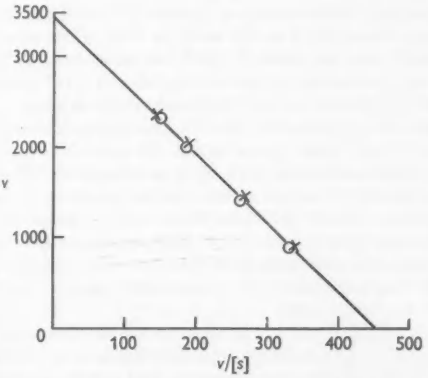

Fig. 7. Comparison of the activity of the cytochrome *c* in two preparations using the cytochrome-oxidase system of heart muscle: (1) the usual Keilin & Hartree (1945) preparation containing 0·34% iron (×), (2) the purest fraction obtained containing 0·465% iron (○). Modified Lineweaver & Burk (1934) type of plot, according to Hofstree (1952); *v* at each concentration of added cytochrome *c* was calculated as μl. O_2/hr./mg. fat-free dry weight of the heart-muscle preparation. [*s*] was taken as the estimated molar concentration of added cytochrome *c* and determined spectrophotometrically. Each flask contained: 0·066 M phosphate buffer pH 7·3, 10^{-3} M ethylenediaminetetraacetic acid, 10 mg. ascorbic acid, 0·376 mg. heart-muscle preparation (fat-free dry weight) and from $2·72 \times 10^{-5}$ M–$16·3 \times 10^{-5}$ M added cytochrome *c*. Total volume of fluid 3·3 ml./flask. Temperature 37°. The O_2 uptake values were obtained by deduction of the O_2 uptake of controls containing, at each concentration of added cytochrome *c*, all the ingredients except the cytochrome oxidase, from that of the complete system.

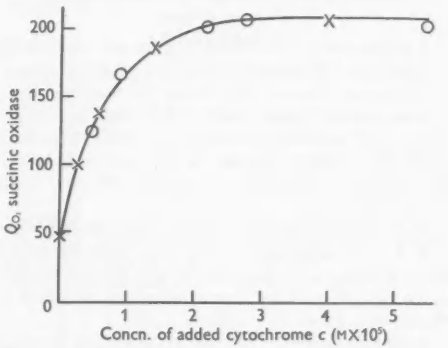

Fig. 8. Comparison of the activity of the cytochrome *c* in two preparations using the succinic-oxidase system of kidney: (1) the usual Keilin & Hartree (1945) preparation containing 0·34% iron (×), (2) the purest fraction obtained containing 0·465% iron (○). Final concentrations in the reaction mixture were: phosphate buffer pH 7·3, 0·10 M; succinate 0·024 M; added cytochrome *c* from 0 to $5·5 \times 10^{-5}$ M (estimated spectrophotometrically). Kidney preparation (fat-free dry weight) 0·865 mg./flask. Total volume 3·3 ml./flask. Temperature 37°. From the O_2 uptake between 5 and 25 min. after adding succinate, Q_{O_2} values were calculated as μl. O_2/hr./mg. fat-free dry weight of the kidney preparation.

same concentration and pH as used for the extraction of the heart-muscle mince transformed part of another chromatographic fraction into fraction II pH 7. This artificially prepared fraction II pH 7 was compared both as regards rate of ascorbic acid oxidation and enzymic activity to the fraction II pH 7 prepared from the 0·34% iron cytochrome *c* preparation. The results presented in Figs. 5 and 6 show they had very similar properties.

Fraction II pH 7 was therefore an artifact produced by the trichloroacetic acid treatment at the time of extraction or possibly during the final precipitation (see procedure for the preparation of horse-heart cytochrome *c*, Keilin & Hartree, 1945). The fraction I pH 7 isolated in the above experiment, in which an artificial fraction II pH 7 was prepared by treatment of fraction I pH 7 with trichloroacetic acid, was found to have activities equal to those of the starting material in both the cytochrome-oxidase and succinic-oxidase systems, and the same rate of ascorbic acid oxidation. Thus, treatment with trichloroacetic acid modified only a proportion of the cytochrome *c*, depending on the concentration of the acid and the time of incubation in its presence. This occurred even under the relatively mild conditions used in the extraction of cytochrome *c* from heart mince. The modified form was easily separable from the native cytochrome *c* as fraction II pH 7.

Maximal purity obtainable by cation-exchange chromatography

A preparation with 0·450% iron, and containing all the chromatographic fractions except the non-cytochrome fraction, was made by washing cytochrome *c* adsorbed on a column of Amberlite IRC-50, with 0·15M ammonium acetate till the cytochrome front was three-quarters of the distance down the column. It was then eluted off with 0·5M ammonia.

This already partially purified sample was subjected to chromatography at pH 7, then fraction I pH 7 was chromatographed at pH 9·6, and finally again at pH 7, as in the experiments recorded in Figs. 1–3.

Analyses showed that, as in experiments using the 0·34% iron preparation, fraction II pH 9·6 (oxidized part) had the highest percentage of iron (0·465%). Table 3 records some of the analytical and spectrophotometric data on this preparation, and Figs. 7 and 8 compare its activity in the cytochrome-oxidase and succinic-oxidase systems with that of the original 0·34% iron preparation from which it was made, and which contained about 27% of non-cytochrome protein. The figures show that there was no significant difference in the enzymic activities of the two preparations per μmole of cytochrome *c* iron.

It should be noted that the preparation containing 0·465% iron does not necessarily represent the purest material obtainable by this method, since the ratio of the optical density at 550 mμ. (reduced) to 280 mμ. (oxidized) was 1·192 (see Table 3), whereas fractions having a ratio of 1·210 were obtained by taking small samples at the peak of the appropriate band during chromatography.

DISCUSSION

The results presented in this paper are in accord with those of Paléus & Neilands (1950), as regards the maximal purity obtainable by chromatography on cation exchangers, and with those of Boardman & Partridge (1953) on the effect of pH and cation concentration on the movement of the bulk of cytochrome *c* preparations on columns of Amberlite IRC-50. However, a study of the enzymic properties of the fractions isolated by chromatography shows that with the exception of fraction II pH 7 all the other fractions have the same activity in both the cytochrome-oxidase and succinic-oxidase systems. Calculated per μmole of cytochrome *c* iron, this activity is the same as that of the usual 0·34% iron preparation which contains about 27% non-cytochrome protein. Calculated per total protein present in the preparation, the activity of the pure cytochrome *c* is 27% greater than that of the 0·34% iron preparation. Thus, the protein impurity present in the 0·34% iron preparation has neither catalytic nor inhibitory effects in the systems studied under the conditions used. Moreover, chromatography on cation exchangers under the conditions employed does not cause any change in the enzymic activity of cytochrome *c* per μmole of cytochrome *c* iron.

Fraction II pH 7 is unique among the chromatographic fractions of cytochrome *c* in showing a high rate of ascorbic acid oxidation and a low enzymic activity in the cytochrome-oxidase system. It causes most of the ascorbic acid oxidation of the 0·34% iron cytochrome *c* preparations and was found to be an artifact of the acid extraction used in the ordinary procedure. The effect of trichloroacetic acid is to change a part of the extracted cytochrome *c* into a modified form, which is entirely indistinguishable spectroscopically from the normal cytochrome *c*, but which can easily be separated from it on cation exchangers, as fraction II pH 7, and has a low enzymic activity. The normal cytochrome *c* (fraction I pH 7) separated from a preparation which has been largely modified by a drastic treatment with trichloroacetic acid is, nevertheless, entirely normal with respect to its enzymic activity in the cytochrome-oxidase and succinic-oxidase systems and its oxidation of ascorbic acid.

Chromatography on cation exchangers causes a gradual decrease of the rate of ascorbic acid oxidation by the successive fractions eluted; this probably represents the separation from the bulk of the cytochrome *c* of a small amount of a modified cytochrome *c* which can act as a very effective catalyst in the oxidation of ascorbic acid. The purer cytochrome *c* fractions have a rate of ascorbic acid oxidation decreased to about 33–20 % of that of the usual 0·34 % iron preparation.

Apart from fraction II pH 7 and the very small fraction I pH 9·6, cytochrome *c* falls entirely into two chromatographic species, reduced and oxidized cytochrome *c*. These two species are reversibly transformable one into the other by reduction and oxidation, their relatively small difference in chromatographic behaviour being entirely due to their state of oxidation. This separation of reduced and oxidized cytochrome *c* on a cation-exchange column, the reduced form acting as the stronger acid, agrees well with the difference of one equivalent in the titration curves of both forms of the protein, observed in the physiological range of pH's, by Theorell & Åkeson (1941). However, a forerunning, reduced fraction may be less pure (lower $E_{550\,m\mu}/E_{280\,m\mu}$ and higher rate of ascorbic acid oxidation) than the following, oxidized fraction (see Table 1).

Reduction of cytochrome *c* was observed during chromatography. It occurs only when the protein is adsorbed on and eluted off solid particles and not in solution. This is, however, not a unique property of the cation-exchange resin used, but occurs also on other adsorbents, such as an aluminium silicate cation exchanger and calcium phosphate gel. The higher the pH at which the adsorption and elution takes place, the higher the percentage of the cytochrome *c* reduced, up to pH 11, where cytochrome *c* becomes rapidly auto-oxidizable. A similar phenomenon has been observed by Paléus (1952) during the electrophoresis of cytochrome *c* preparations on paper.

SUMMARY

1. Native horse-heart cytochrome *c* shows only two chromatographic species on cation exchangers, reduced and oxidized cytochrome *c*. These two chromatographic species are reversibly transformable one into the other. Other fractions are artifacts of the preparation.

2. Trichloroacetic acid modifies a variable proportion of any sample of cytochrome *c* into a form indistinguishable spectroscopically from the normal cytochrome *c*, but easily separated from it on cation-exchange columns, and having a low enzymic activity, and a high rate of ascorbic acid oxidation.

3. The degree of reduction of ferricytochrome *c* on adsorption and elution from a number of adsorbents increases with the pH up to 11, where cytochrome *c* becomes rapidly auto-oxidizable.

4. The rate of ascorbic acid oxidation of various cytochrome *c* preparations varies inversely with their degree of purity.

5. The enzymic activity per μmole of cytochrome *c* iron of the purest preparation obtained (0·465 % iron), both in the succinic-oxidase and the cytochrome-oxidase systems, is the same as that of the 0·34 % iron type of preparation containing about 27 % non-cytochrome protein, the non-cytochrome protein impurity having neither catalytic nor inhibitory effects in the systems and under the conditions used.

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Some Effects of Vitamin B_6 Deficiency on Fat Metabolism in Rats

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In a number of studies on the role of vitamin B_6 in fat metabolism in the rat, attention has been given to the effect of the vitamin deficiency on the content of fat in the liver, carcass, blood and kidneys. Data on the composition of liver with respect to total fatty acids, neutral fat, cholesterol or phospholipids have been provided by the work of several investigators; the literature has been reviewed by Carter & Phizackerley (1951). Comparison of results obtained by the various workers regarding the lipotropic property of pyridoxine is difficult because some of the diets were incomplete with respect to lipotropic factors. Restricted or liberal supply of food may also be a cause for the variation in the data. Experiments on the total lipid concentration of the carcass as affected by pyridoxine have yielded consistent results when fat-free diets were fed. McHenry & Gavin (1938, 1941) showed that, on a fat-free diet, pyridoxine-supplemented animals have a higher content of body fat than deficient groups. This was so whether the supplemented groups were fed *ad lib.* or were pair-fed with the vitamin-deprived groups. When fat was included in the diet, results have been conflicting. Carter & Phizackerley (1951) could not detect a statistically significant difference in the body fat between deficient and control groups. Greater unsaturation of carcass fat, as measured by its iodine value, has been found in pyridoxine deficiency (Quackenbush & Steenbock, 1942). The total lipids in the blood, liver and kidneys of deficient animals had a lower iodine value (Tulpule & Patwardhan, 1950).

The work described in this paper relates to the role of pyridoxine in the metabolism of fat in the rat. The content and fatty acid distribution of the body fat of pyridoxine-deficient rats as compared with pair-fed controls has been investigated. The ability of the pyridoxine-deficient rat to convert ^{14}C-labelled glucose into fatty acids has been studied. Since it has been demonstrated (Quackenbush, Steenbock, Kummerow & Platz, 1942) that linoleic acid and pyridoxine have a complementary action in the cure of acrodynia, the effect of supplementation with linoleic acid *per se* and in corn oil has also been studied.

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EXPERIMENTAL

Albino rats of the Wistar strain were used throughout, and they were housed individually in screen-bottom cages with water provided *ad lib.* There were two diets with the following compositions:

(*a*) *Casein diet.* This consisted of vitamin- and fat-free casein, 90; vitaminized casein, 4; agar, 2; salt mixture (Steenbock & Nelson, 1923), 4; choline chloride, 0·2; inositol, 0·2%.

(*b*) *Sucrose diet.* This consisted of sucrose, 74; vitamin- and fat-free casein, 16; vitaminized casein, 4; agar, 2; salt mixture, 4; choline chloride, 0·2; inositol, 0·2%.

Vitaminized casein was obtained by mixing into 800 g. vitamin- and fat-free casein the following: thiamine hydrochloride, 100 mg.; riboflavin, 100 mg.; calcium pantothenate, 400 mg.; nicotinic acid, 400 mg.; *p*-aminobenzoic acid, 400 mg.; biotin, 20 mg.; pteroylglutamic acid, 20 mg.

Preparation of tissue lipids. Livers were extracted by the procedure of Carter & Phizackerley (1951), and phospholipids were separated by initial treatment with acetone followed by $MgCl_2$. Bodies from each group of rats were frozen in liquid air and ground to a fine paste in a power mincer. After samples had been removed for determination of moisture and of nitrogen, the balance of the pooled carcasses was dehydrated with cold ethanol. Fat was extracted with boiling ethanol and then with ether in a percolator. The combined extracts were concentrated *in vacuo*, the residue mixed with light petroleum, and the mixture was filtered after standing overnight. A sample of the filtrate was taken for phosphorus estimation and the balance was freed of solvent by evaporation. The residue was extracted with acetone and a conventional $MgCl_2$ precipitation was carried out to separate phospholipids. Neutral fat and unsaponifiable material were recovered from the acetone filtrate.

Analytical methods. Total nitrogen was determined by the Kjeldahl method. Moisture was estimated by drying tissue samples to constant weight at 110°. Phosphorus was determined by the procedure of Fiske & SubbaRow (1925), and the content of phospholipids calculated on the assumption that rat phospholipids contain 4% P. Iodine number was determined by the method of Yasuda (1931). Fatty acids were separated as lead salts by the procedure of Hilditch (1949). Fractionation of methyl esters of fatty acids was done in a 50 ml. distilling bulb using a copper coil as a fractionating column as described by Lovern (1934).

Animals on casein diet

Sixty rats, having an initial average weight of 120 g., were divided into six groups comparable with regard to average weight and sex and they were maintained on the casein diet.

Table 1. *Composition of liver and of carcass of rats fed on a casein diet*

Group and no. of animals	Supplements	Average wt. (g.)		Average food intake (g./rat/day)	Moisture (%)		Protein* (%)		Total lipids (%)		Phospholipids (%)		Iodine value of total lipids	
		Liver	Carcass		Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass
A (10)	None	5·3	79	6·2	72	70	21·8	24·1	4·8	1·0	2·1	0·73	67	81
B (10)	Pyridoxine	6·7	122	6·2	72	68	20·7	22·7	5·1	2·7	2·5	0·54	61	64
C (9)	Linoleic acid	6·0	77	6·2	71	71	20·7	23·1	5·2	1·9	2·4	1·48	71	72
D (10)	Pyridoxine + linoleic acid	5·8	121	6·2	73	69	20·8	22·4	4·9	3·9	2·4	0·69	67	55
E (8)	8 weeks, none; last 4 weeks, pyridoxine	9·0	149	8·1	72	67	23·5	21·2	4·7	2·5	2·4	0·28	66	66
F (8)	12 weeks, pyridoxine	8·7	146	8·1	72	68	22·7	21·6	4·8	3·2	2·3	0·31	64	69

* $N \times 6{\cdot}25$.

Table 2. *Composition of liver and of carcass of rats fed on a sucrose diet*

Group and no. of animals	Supplements	Average wt. (g.)		Average food intake (g./rat/day)	Moisture (%)		Protein* (%)		Total lipids (%)		Phospholipids (%)		Iodine value of total lipids	
		Liver	Carcass		Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass	Liver	Carcass
N (10)	Normal stock diet	—	110	—	—	63	—	18·5	—	7·3	—	0·61	—	79
1 (9)	None	7·3	142	9·7	72	66	18·9	21·4	1·3	4·6	1·8	0·54	75	64
2 (9)	Pyridoxine	6·8	174	9·7	71	63	19·6	19·2	1·2	8·9	1·9	0·36	65	61
3 (9)	Linoleic acid	5·5	141	9·7	72	67	21·1	20·6	1·1	6·1	2·2	0·48	66	64
4 (9)	Pyridoxine + linoleic acid	6·9	164	9·7	70	64	21·9	19·7	1·4	8·3	2·8	0·30	65	62
5 (9)	None, 16 weeks	6·9	106	8·2	73	68	19·2	22·1	2·0	3·6	1·8	0·62	59	72
6 (9)	Pyridoxine	6·7	168	8·2	74	66	19·4	21·4	1·0	8·2	1·9	0·56	65	66
7 (5)	None	7·8	168	8·2	72	66	20·2	22·3	1·1	6·8	2·4	0·74	86	92
8 (5)	Pyridoxine	6·5	215	8·2	71	62	20·1	21·9	1·4	12·6	3·0	0·64	91	91

* $N \times 6{\cdot}25$.

Four groups were given for 8 weeks the following treatment: *A*, no supplement; *B*, 50 μg. pyridoxine hydrochloride/rat/day; *C*, 20 mg. linoleic acid/rat/day; *D*, same amounts of pyridoxine and of linoleic acid. At the end of 8 weeks, animals in these groups were killed for analysis. Groups *B–D* were fed isocalorically with group *A*, which was fed *ad lib.* In addition there were two more groups, *E* and *F*, which received the same treatment as *A* and *B*, respectively, but the treatment was continued for 12 weeks; during the last 4 weeks group *E* was given the pyridoxine supplement. Group *F* was pair-fed with *E*. After 12 weeks animals in groups *E* and *F* were killed for analysis. It had been intended originally to give deoxypyridoxine to all rats in order to expedite the deficiency state and 100 μg. desoxypyridoxine hydrochloride/rat/day was included in all feedings. After 1 week the antivitamin was found to have reduced food consumption drastically and its use was stopped. Analytical data for all groups are given in Table 1.

Animals on sucrose diet

Rats with initial weight 120–130 g. were maintained on the sucrose diet to provide a comparison with those given the casein diet. Groups 1–4 had the same respective treatment as *A–D* (casein diet) but were maintained for 10 weeks before killing for analysis. Group 5 was continued for 16 weeks without supplement to determine the effect of prolonged deficiency and group 6 was a pair-fed, pyridoxine-supplemented control for group 5.

To determine the effect of the inclusion of a reasonable amount of fat in the basal diet, two groups were maintained for 10 weeks on the basal sucrose diet to develop a deficiency state characterized by acrodynia and were fed for 6 subsequent weeks on a diet in which 15% by weight of sucrose was replaced by an equal weight of corn oil. Group 7 was deprived of pyridoxine throughout the entire period, while group 8 was supplemented with pyridoxine and pair-fed with group 7 during the last 6 weeks. These two groups were killed for analysis at the end of the 16th week.

Analytical results for groups 1–8 are shown in Tables 2 and 3. Both tables include a group of rats of the same weight as the initial weight of groups 1–8 but which had received no experimental diet or treatment. This group is designated as *N*.

Administration of [^{14}C]*glucose*

Three groups of rats, six animals/group, with an average initial weight of 100 g., were fed the sucrose diet for 6 weeks. Group I received no pyridoxine and all rats in this group had acrodynia in the sixth week. Each rat in groups II and III received 50 μg. pyridoxine hydrochloride/day. Group II was pair fed with group I, and group III was fed *ad lib.* All rats received 100 μg. desoxypyridoxine/day. During the six weeks the rats were habituated to insertion of a stomach tube.

After 6 weeks all rats received by stomach tube 1 ml. solution containing 60–75 mg. [^{14}C]glucose. The labelled glucose was kindly supplied by Prof. G. Krotkov, Queen's

Table 3. *Distribution of fatty acids in the carcass fat, sucrose diet*

Values represent the individual fatty acids as a percentage of the mixed fatty acids.

Group and no. of animals	Supplements	Saturated fatty acids				Unsaturated fatty acids			
		C_{14}	C_{16}	C_{18}	C_{20}	C_{16}	C_{18}	C_{18} dienoic	C_{20-22}
1 (9)	None	2·6	19·4	1·1	—	13·3	58·3	2·5	2·8
2 (9)	Pyridoxine	3·4	23·8	2·2	—	13·2	56·6	0·8	0·6
3 (9)	Linoleic acid	1·8	22·0	0·8	—	20·1	52·1	3·1	Trace
4 (9)	Pyridoxine + linoleic acid	1·9	24·1	0·7	—	19·1	49·1	3·6	—
5 (9)	None	8·1	21·5	—	—	30·2	36·1	4·1	—
6 (9)	Pyridoxine	7·3	20·7	—	—	30·0	39·3	2·8	—
7 (5)	Corn oil	6·2	10·1	—	—	45·4	27·3	11·0	—
8 (5)	Pyridoxine + corn oil	6·0	11·1	—	—	45·6	27·5	11·9	—
N (10)	Normal on stock diet	1·6	19·6	2·5	1·8	4·3	55·9	12·2	2·1

Table 4. *Incorporation of* ^{14}C*-labelled glucose into rat-carcass fat*

Group I (deficient)*			Group II (pair-fed control)*			Group III (control fed *ad lib.*)†		
Wt. of rat (g.)	Activity of glucose fed (counts in million/min.)	Incorporation of activity into fat (%)	Wt. of rat (g.)	Activity of glucose fed (counts in million/min.)	Incorporation of activity into fat (%)	Wt. of rat (g.)	Activity of glucose fed (counts in million/min.)	Incorporation of activity into fat (%)
80	4·78	0·49	110	4·78	0·28	172	4·78	0·42
100	4·78	0·45	110	4·78	0·24	217	4·66	0·31
80	4·66	0·38	115	4·78	0·38	257	4·66	0·28
102	4·66	0·39	117	4·66	0·35	257	4·66	0·37
128	4·66	0·82	136	4·66	0·42	190	2·88	0·35
100	2·88	0·35	140	4·66	0·46	210	2·88	0·35
	Average	0·48 ± 0·17‡			0·36 ± 0·08‡			0·35 ± 0·05‡

The '*t*' values for significance at the 5% level were 1·51 between groups I and II, and 1·75 for groups I and III.

* Average daily food intake 7·2 g./rat.
† Average daily food intake 13·4 g./rat.
‡ Values represent S.D. for the data in the group.

University, Kingston, Canada. The glucose had been prepared by a photosynthetic method using tobacco leaf and $^{14}CO_2$; it contained ^{14}C equally in all positions, and it had an activity of 1·21 mc/g. During the 26 hr. after glucose administration the above feeding regimen was continued and the animals were kept in vacuum-type desiccators to which CO_2-free oxygen/nitrogen was passed and from which CO_2 was absorbed in 10% KOH solution. After the 26 hr. period the rats were killed by stunning, and neutral fat samples were prepared from each rat. After measurement of activity in the individual fat samples, pooled samples of fat, obtained by mixing one-fourth of the fat extracted from each rat in a particular group, were hydrolysed and the unsaponifiable matter as well as the free fatty acids were recovered for radioactivity measurements. The fatty acids were separated into solid and liquid lead-salt fractions and the activity in the liquid lead-salt portion was determined. The activity of the original glucose solution was checked. Data from the experiment with labelled glucose are given in Table 4.

DISCUSSION

The total amount of fat in the livers of rats fed the casein diet was greater than in the livers of rats maintained on the sucrose diet, but deprivation of pyridoxine had no observable effect upon liver fat and in no case was there sufficient fat to warrant designation of a liver as fatty. A supply of linoleic acid did not apparently alter the quantity or quality of liver lipids.

All rats fed the casein diet had less carcass fat than did rats on the sucrose diet and this may have been due to a difference in food consumption. With both diets, lack of pyridoxine in the diet caused a decrease in the percentage of total fat in the carcass, an effect which was partially prevented by a supply of linoleic acid. The proportion of phospholipids and the iodine value of total lipids were greater in deprived than in control animals. The data suggest that vitamin B_6-deficient rats conserve phospholipids and unsaturated fatty acids.

Since the experimental diets were basically free of fat, it could be concluded that the control rats, particularly those on the sucrose diet, were able to synthesize fat. However, the markedly different amounts of fat in deprived and control animals cannot be taken as evidence that fat synthesis did not occur since an increase in fat storage may have been prevented by rapid utilization. The experiment with labelled glucose indicated that the incorporation of ^{14}C into carcass lipids was at least as much in deficient as in control rats. However, the amount of incorporation was small and it is inadvisable to draw a definite conclusion.

SUMMARY

1. Using isocaloric feeding of two fat-free basal diets, one high in protein and the other in carbohydrate, rats supplied with pyridoxine showed an increase in carcass fat with a decreased iodine value.

2. Rats deprived of vitamin B_6 contained markedly less carcass fat with an apparent conservation of phospholipids and of unsaturated fatty acids. There were no marked differences in the amount or characteristics of liver lipids. Data on the incorporation of ^{14}C (from fed glucose) suggest that fat synthesis proceeded equally in control and deficient rats. The decreased quantity of fat in deprived rats may have been due to rapid utilization.

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Determination of Monoamino Monocarboxylic Acids by Quantitative Paper Chromatography

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Many methods for chromatographic separation of amino acids are now available, but their subsequent determination, generally employing ninhydrin, often leaves much to be desired. It was felt, therefore, that there was a need for a simple method of analysis based on the convenient paper technique and employing some means of determination less fickle than ninhydrin colorimetry.

The method now presented involves an initial one-dimensional separation of amino acids in considerable quantity, using bands rather than spots. The problem of accurate marking of these bands, with little space between them and often having irregular fronts, has been solved by spraying with an indicator solution of bromothymol blue, formaldehyde and alkali, in ethanol (Kemble & Macpherson, 1952). This restricts the range of solvents to those neutral in character, but several such are known to give useful separations of amino acids. Methods of determination were then investigated. Measurement of carbon dioxide following oxidation with ninhydrin is a most specific and accurate method (Van Slyke, Hamilton, McFadyen & Dillon, 1941), but note was taken, in the same paper, of the use of chloramine-T for decarboxylation at low temperatures. Cohen (1940) has measured the evolution of carbon dioxide by chloramine-T in a Warburg respirometer. As numerous determinations can be performed simultaneously, this apparatus was adopted for the present work. A 9 in. wide strip of Whatman 3MM paper will separate at least 2 mg. of each amino acid, sufficient to give a maximum reading on the manometer.

Recoveries of carbon dioxide from amino acids obtained by Krebs (quoted by Cohen, 1940) are significantly higher than the more nearly theoretical values reported by Van Slyke *et al.* (1941). Preliminary work showed that yields of carbon dioxide following direct oxidation with chloramine-T were in accord with the results of Krebs, while yields obtained from a sprayed chromatogram agreed with those of Van Slyke. This difference was found to be due to the suppression by the formaldehyde of the indicator spray of interference due to ammonia, and all recovery factors (moles CO_2/mole amino acid) have been determined in the presence of a standard amount of formaldehyde.

Work has been confined in the present instance to the monoamino monocarboxylic acids, which can be isolated readily by ionophoresis in a three-compartment cell.

METHODS

Chloramine-T reaction

Standard solutions of amino acids. DL-Amino acids were used with the exception of glycine, L-hydroxyproline and L-tyrosine. In all cases paper chromatograms showed no ninhydrin-reacting impurities to be present, and micro-Kjeldahl determinations showed the nitrogen contents to be 99–100% of the theoretical value.

Standard aqueous solutions of the amino acids were prepared of strength 1–3 mg./ml.

Warburg constant volume respirometer. The temperature of the bath was maintained at 30° and the manometer fluid was prepared as described by Krebs (1951).

For each set of determinations a thermobarometer was used in addition to the manometer for determining the chloramine-T blank and no more than three manometers were read in conjunction with the thermobarometer at any one time. Thus errors due to minor fluctuations of temperature and pressure are avoided.

Determination of recovery factors. The recovery of CO_2 from amino acids was independent of the amount of formaldehyde present over the range 0·1–0·2 ml. formalin (Table 1) and was almost independent of the amino acid concentration. All recovery factors, therefore, were established in presence of 0·15 ml. commercial formalin (37–41% (w/v) aqueous formaldehyde), and the Warburg flasks were prepared by adding a measured quantity of amino acid solution, 0·4 ml. buffer (8·24 g. trisodium citrate and 76·6 g. citric acid dissolved in 250 ml. water, pH 2·5), 0·15 ml. formalin, and water up to a final volume of 2·5 ml. 0·5 ml. aqueous chloramine-T solution (12%, w/v) was added to the side arm.

Table 1. *Moles of gas, measured as* CO_2, *evolved from one mole of amino acid by chloramine-T in the presence of varying amounts of formaldehyde at* pH 2·5 (*see text*)

	Quantity of formalin (ml.)					
Amino acid	0	0·05	0·10	0·15	0·20	0·50
Alanine	1·10	0·99	0·98	0·99	0·99	1·00
Glycine	1·26	0·99	0·99	0·96	0·98	0·90
Leucine	1·08	1·01	1·00	1·02	1·00	0·98
Phenylalanine	1·04	0·99	0·93	0·93	0·92	—
Valine	1·09	1·01	1·03	1·03	1·00	0·97

Table 2. *Moles CO_2/mole amino acid evolved by chloramine-T for various amino acid concentrations at pH 2·5 in presence of 0·15 ml. formalin (see text)*

	Wt. of amino acid (mg.)					
Amino acid	2·19	1·42	1·10	0·81	0·73	0·40
Serine	—	0·951±0·004	—	0·925±0·012	—	0·897±0·011
Threonine	—	0·918±0·009	—	0·891±0·011	—	0·877±0·018
Valine	1·02±0·01	—	1·03±0·01	—	1·00±0·01	—

Table 3. *Recovery factors (moles CO_2/mole amino acid) for individual amino acids in the reaction with chloramine-T at pH 2·5 (see text)*

		Moles CO_2/mole amino acid			
Amino acid	Weight range investigated (mg.)	Van Slyke *et al.* (1941)	Present work	Mean variation	No. of determinations
Alanine	1·52–0·46	1·03	0·997	0·006	5
Glycine	1·02–0·26	0·972	0·969	0·006	5
Hydroxyproline	2·54–0·51	—	0·971	0·011	7
Isoleucine	1·91–0·38	—	1·021	0·008	5
Leucine	1·98–0·40	1·017	1·018	0·007	5
Methionine	1·62–0·65	—	0·864	0·009	10
Phenylalanine	2·05–0·51	0·946	0·931	0·006	5
Proline	1·68–0·50	—	0·904	0·007	6
Serine	1·42–0·40	1·028	0·951–0·901	0·004–0·011	3×5
Threonine	1·38–0·39	1·015	0·918–0·873	0·009–0·008	3×5
Tryptophan*	0·77	0·751	0·33	—	1
Tyrosine†	2·67–0·67	1·033	0·980	0·012	5
Valine	1·98–0·40	—	1·023	0·008	5

* The brown precipitate, observed by Van Slyke *et al.* (1941) when chloramine-T was added to tryptophan, was not obtained in the presence of formalin.

† Solution made up in citrate buffer (pH 2·5).

Table 4. *Evolution of gas, measured as CO_2, in reaction between 0·585 mg. NH_3 and chloramine-T in 20 min. (see text)*

	pH 2·5		pH 4·7*	
	No formalin	Formalin (0·2 ml.)	No formalin	Formalin (0·2 ml.)
Gas evolved (μl. at s.t.p.)	484·3	15·6	650·1	244·1

* 0·4 ml. buffer (35·3 g. trisodium citrate and 16·8 g. citric acid dissolved in 150 ml. water) was used.

With the exception of proline and hydroxyproline, when 100 min. was needed, gas evolution was complete within 20 min. and final manometer readings were taken accordingly.

For each amino acid at least five determinations were made on different quantities of standard solution, and the yield of gas was found to be independent of amino acid concentration except for serine and threonine. With these the variation amounted to little more than 5% over the range useful for estimation (Table 2).

The appropriate recovery factors listed in Table 3 are applied for all calculations made throughout the remainder of this paper except in the case of tryptophan, where the factor was found to be low and irregular.

Reaction between chloramine-T and substances other than α-amino acids. Chloramine-T is known to form nitrogen from ammonia (Cohen, 1940) and this reaction was investigated. The evolution of gas from ammonia was almost completely inhibited by formaldehyde at pH 2·5 (Table 4).

Primary and secondary amines also evolve gas with chloramine-T but will be removed from the neutral amino acids during ionophoresis.

Under the conditions of analysis, tertiary amines, pyridine, urea, γ-aminobutyric acid and fructose gave no evolution of gas with chloramine-T.

No observations have been made on peptides but at the higher temperature of 45° Van Slyke *et al.* (1941) reported values up to 0·075 moles CO_2/mole for several dipeptides.

Analysis of made-up mixtures

Chromatography tanks. These consisted of glass jars 21 in. deep, of area 11·5×7·5 in. and having a glass cover. The troughs were Alkathene tubes (1·5 in. bore) with a 0·8 in. wide slit cut to within 0·5 in. of the ends. They were held in position by pressure of the rubber bungs sealing each end against the walls of the jar.

Preparation of papers. Whatman 3MM paper 9 in. wide was used with 18·5 in. from the starting line to the bottom of the strip. A margin was ruled down each side, 0·5 and 0·7 in. from the respective edges.

Chromatograms run with propanol:water, when sprayed with ninhydrin, exhibited pinkish 'beards' in front of the spots; the inclusion of cupron in the solvent (Consden, Gordon & Martin, 1944), and even washing papers with an ethanolic solution of cupron before use did not entirely prevent the condition. The following treatment, however, proved successful. A solution of 8-hydroxyquinoline (30 mg. in 1 ml. acetone) was applied along the intended starting line and the paper was then eluted chromatographically with acetone until the reagent colour was

washed from the bottom of the sheet. This took 3–4 hr., and after drying at room temp. the paper was ready for use.

The solution for analysis, containing 1–2 mg. α-amino N and of volume preferably not greater than 2 ml., was applied as a band along the starting line using an 'Agla' micrometer syringe with a glass needle (Burroughs, Wellcome and Co. Ltd., London). The band was kept well within the limits defined by the ruled margins. After each addition most of the solvent was driven off, using warm air from a hair drier, before more solution was applied. The band was not allowed to exceed 0·5 in. in width. Two identical papers were prepared, to be developed in the solvents described below. They were folded 1·8 in. above the starting line before insertion into the trough and held in position by a glass rod in the bottom of the trough.

Solvents. Many solvents have been described in the literature for the separation of amino acids but, bearing in mind the need for a neutral solvent, the mixture propanol: water (80:20 by vol.) has proved the most useful of those tested.

On a Whatman 3MM paper 18 in. long the following separation was achieved in 32–36 hr. at room temp.: glycine + serine; threonine + hydroxyproline; alanine; proline + tyrosine + tryptophan; valine + methionine; phenylalanine; leucine + isoleucine (order of increasing R_F).

Investigations were carried out to try to find another solvent mixture of different properties in which a second chromatogram could be run and used in conjunction with the one obtained from propanol: water. The most useful of the mixtures tried was *iso*butanol: methyl ethyl ketone: water (70:50:30 by vol.) which gave the following separation on an 18 in. strip of Whatman 3MM paper in 40–44 hr. at room temp.: glycine + serine; threonine + hydroxyproline + alanine; proline; valine; methionine + tyrosine; isoleucine; leucine partially separated from phenylalanine and tryptophan (order of increasing R_F).

Detection and elution of amino acid bands. After the two chromatograms have run for the requisite time they are removed from the tanks and hung in the laboratory for some minutes. They are not allowed to become quite dry, however, as the indicator spray seems most effective if the paper is still slightly moist with solvent.

The indicator spray, as previously described (Kemble & Macpherson, 1952), has had its formalin content decreased in order that the contribution of the spray to the amount of formaldehyde in the flasks before analysis shall be negligible. A 1% (v/v) content of formalin was found to be sufficient to allow the spray to function satisfactorily. Thus the spray consists of 20 ml. 0·15% (w/v) solution of bromothymol blue in 95% (v/v) aqueous ethanol, 0·2 ml. formalin and 0·2 ml. 20% (w/v) aqueous KOH. The optimum content of the last constituent has been found to vary with different batches of paper.

Using an apparatus giving a fine mist, the chromatogram is sprayed from the top down and, as the position of each band of amino acid is made apparent, it is marked with a ball-point pen before spraying is continued.

When the chromatogram has dried, the strips containing the amino acid are cut out for elution. The 0·7 in. margin is cut in the shape of a V leaving a tail 0·2 in. long and 0·1 in. wide at its point. A fold is made along the 0·5 in. margin and the fold hung over the lip of a small, brass trough and held in position by a microscope slide. The trough, which can be adjusted for height, can accommodate 10–12 strips, which draw water out of the trough and down the paper in the manner of a chromatogram. The water finally collects in the paper tail at the bottom of the strip and drips off into a tared Warburg flask or a 5 ml. graduated flask suitably placed beneath. The whole apparatus is covered by an inverted glass tank to prevent excessive evaporation of water during elution.

It has been found that when all the indicator has been washed from the paper (about 2 ml. water), then all the amino acid will also have been eluted.

Estimation. After elution the Warburg flasks are weighed to ascertain the volume of the contents.

The eluate from the (threonine + hydroxyproline + alanine) strip of the *iso*butanol chromatogram is used to determine whether or not hydroxyproline is present. This solution is shaken with 1·0 ml. butanol and the butanol layer, which will now contain the indicator, is discarded. A test for hydroxyproline is carried out on the aqueous layer after the method of Neuman & Logan (1950), using *p*-dimethylaminobenzaldehyde. If hydroxyproline is absent then the eluate from the threonine strip of the propanol: water chromatogram is directly oxidized with chloramine-T.

Should the volume of solution be greater than 2·0 ml. after elution, then the flask constant can be adjusted by use of the equation: $K_1 = K - 0{\cdot}0236\Delta V$, where K is the constant established for a total final vol. of 3 ml. and ΔV ml. is the vol. of eluate in excess of 2·00 ml. This equation also shows that a balance weighing to 0·1 g. is sufficiently accurate to weigh the flask and flask contents since, assuming an average value of 1·700 for K, an error of 0·1 g. in the weight of the flask contents implies a negligible error (0·1%) in the subsequent analysis.

If the strip has been eluted into a 5 ml. flask, as always with glycine + serine and threonine + hydroxyproline mixtures, then a known sample is transferred to a Warburg flask.

Subsequent treatment is exactly as has been described for standard amino acid solutions.

Blanks. The ammonia content of the paper, which has caused so much trouble with ninhydrin-colorimetric determinations, presents no problem in these estimations. The amount of ammonia which can be washed from a sprayed strip of 3MM paper 9 × 2 in. has never been found to exceed 0·05 mg. and thus can be neglected as a contributor towards total gas evolution (Table 4).

There remains the possibility of other contaminants reacting with chloramine-T, but during a series of determinations made on blank chromatograms run in neutral solvents the volume of gas evolved from the eluates never exceeded that of the chloramine-T blank by more than 2 µl. However, it is necessary to distil commercial *iso*butanol from acid solution before use.

Thus the only blank determination which has to be made is on the chloramine-T solution.

Mutual effect of two amino acids on respective CO_2 *evolutions.* Since no known solvent will completely separate all the amino acids, it is sometimes necessary to estimate the total evolution of gas from a mixture. For example, using propanol: water (80:20, v/v) as solvent, serine and glycine cannot be separated. However, determinations carried out for weight ratios ranging from 4:1 to 1:4 showed that the amount of gas evolved by each amino acid is not affected by the presence of the other.

Glycine + serine and hydroxyproline + threonine bands. As has been stated, these pairs of amino acids are not separated by the solvents employed, and additional treatment is necessary for their determination.

Table 5. *Recovery of glycine following periodate oxidation of glycine + serine mixtures (see text)*

Time in desiccator	Mixture (mg.) Serine	Glycine	Gas evolved (μl. at s.t.p.)	Recovery (%)
30 min.	1·35	—	−2·1	—
	1·35	0·25	73·6	99·7
	1·35	0·51	150·8	102·2
	0·54	0·51	143·3	97·1
	—	0·51	147·1	99·7
Overnight	1·35	—	−3·6	—
	1·35	0·25	55	74·5
	1·35	0·51	134·8	91·3
	—	0·51	149·8	101·5

Table 6. *Recovery of hydroxyproline following periodate oxidation of threonine (see text)*

Time of periodate oxidation (min.)	Mixture (mg.) Threonine	Hydroxyproline	Gas evolved (μl. at s.t.p.)	Recovery (%)
0·25	1·37	—	4·8	—
0·25	—	2·03	335·3	99·2
1·0	—	2·03	336·1	99·4
5·0	—	2·03	303·5	89·8
10·0	—	2·03	283·0	83·7
0·5	1·37	—	6·5	—
0·5	1·37	2·03	335·2	99·2
0·5	—	2·03	334·8	99·0
0·5	1·97*	—	179·3	—

* From chromatogram.

Table 7. *Recovery of hydroxyproline following removal of formaldehyde and periodate oxidation of threonine (see text)*

Mixture (mg.) Threonine	Hydroxyproline	Gas evolved (μl. at s.t.p.)	Recovery (%)
1·48	—	6	—
0·59	0·51	83·7	98·9
0·59	1·02	166·7	98·5
0·30	0·51	80·7	95·4
—	1·02	167·5	99·0

Serine and threonine are oxidized by a neutral or alkaline solution of periodate to give ammonia, glyoxylic acid and the appropriate aldehyde (Nicolet & Shinn, 1939), and determination of the β-hydroxy α-amino acid content of these mixtures was attempted by measurement of the ammonia formed. This was unsuccessful, low and erratic recoveries being obtained by microdiffusion or by aeration. It has been found possible, however, to destroy the serine or threonine with periodate and determine the remaining amino acid by chloramine-T.

(*a*) *Glycine + serine mixtures.* A sample of the glycine + serine solution, containing up to 1·2 mg. glycine, is pipetted into a tared Warburg flask and made to 2 ml. with distilled water. 0·2 ml. 2N-NaOH is added, followed by 0·3 ml. 0·5M periodic acid and the flask gently shaken to ensure thorough mixing. After 1 min., 0·1 ml. ethylene glycol is added and mixed by shaking to ensure destruction of excess of periodate. 0·1 ml. conc. H_2SO_4 is now put in the centre well of the flask which is placed in a vacuum desiccator for 30 min. The acid is then removed from the well and the solution adjusted to pH 7 with 2N-H_2SO_4, after which the flask is weighed to ascertain the volume of its contents, and the glycine is determined in the usual way with chloramine-T. A blank determination on 2 ml. water with the reagents is carried out simultaneously. When the glycine exceeds 0·3 mg., recoveries of 100±3% are obtained (Table 5), but it is important that the flask should not remain in the desiccator for more than 30 min., which period is sufficient to remove the ammonia, since it was found that when left overnight, low recoveries of glycine from glycine + serine mixtures occurred (Table 5). Glycine alone is unaffected so the loss must be due to its reaction with one of the oxidation products of serine, presumably glyoxylic acid.

The accuracy of the serine estimation, arrived at by subtracting the glycine value from that obtained for the glycine + serine mixture, depends on the amount present. Where this is greater than 0·4 mg., recoveries are 100±5%.

The procedure described is applicable to the eluate of the glycine + serine band after spraying with the indicator spray, provided periodate oxidation is performed immediately after elution. When the solutions were kept for some days, the oxidation of serine was incomplete. Experiment showed that this inhibitory effect was due to formaldehyde and that inhibition was markedly less in alkaline than in neutral solutions. The eluate from a sprayed strip is normally slightly alkaline, hence the satisfactory results on immediate periodate oxidation. As a precaution, however, the glycine + serine strip is lightly sprayed with 2N-NaOH before eluting and the eluate oxidized immediately.

(*b*) *Threonine + hydroxyproline mixtures.* The procedure described for glycine + serine mixtures is also applicable to threonine + hydroxyproline solutions, but the time allowed for periodate oxidation is decreased to 30 sec. as hydroxyproline also seems to be attacked, although much more slowly (Table 6).

When applied to eluates from sprayed chromatograms, the inhibitory effect of formaldehyde on periodate oxidation is much more marked with threonine than with serine and it is not possible to get anything approaching complete oxidation no matter how soon after elution the periodate is added (Table 6, last entry). The following procedure, however, seems to break up the threonine-formaldehyde complex. The Warburg flask containing the threonine + hydroxyproline is made approx. 2N with respect to HCl and allowed to remain on a boiling-water bath until thoroughly dry. If the contents are now dissolved in water and treated with periodate, as described above, the threonine is oxidized completely and hydroxyproline can be determined (Table 7). These experiments indicate a recovery of 100±5%.

The accuracy of the threonine value, determined by difference, will depend on the relative amounts of threonine and hydroxyproline present. Except in gelatin and related products, hydroxyproline is normally present in small amount or is absent altogether. In the latter case, a direct determination of threonine can be made to within 5% and in the former, accuracy will be within 8%.

Analysis of natural products

Ionophoresis. In extracts of natural products the free amino acids will be contaminated with salts which must be removed before chromatography and this can be done by ionophoresis in a three-compartment cell (Macpherson,

1946), the basic and acidic amino acids being removed at the same time.

The extract or protein hydrolysate containing up to 20 mg. N is adjusted to pH 6 with dil. NH_3 and placed in the centre compartment of a three-compartment cell with water in the outside compartments. Direct current is passed and migration of charged substances occurs. As this migration nears completion the current will fall and a minimum value (20–40 mA in the apparatus used) will be reached when migration is complete. The centre-compartment solution, which will contain a large proportion of the monoamino monocarboxylic acids, is removed and the contents of the two outside compartments are mixed, adjusted to pH 6 with dil. NH_3 and again subjected to ionophoresis. This process is repeated.

A mixture of the three centre-compartment solutions will now contain all the monoamino monocarboxylic acids originally present.

Hydrolysis of egg albumin and insulin. The procedure used was as follows. Up to 300 mg. protein were dissolved by warming with 30–40 ml. 10N-HCl (A.R.) and the solution was made up to 50 ml. with 10N-HCl. 1 ml. samples were taken for determinations of total N and the remainder of the solution was made up to 500 ml. 6N-HCl and refluxed for 24 hr. The hydrolysate was taken to dryness under reduced pressure, redissolved and filtered to remove humin. The filtrate was made up to 50 ml. and a sample taken for determination of total N and ammonia N. The remainder was subjected to ionophoresis as described above.

No interference on chromatograms due to cysteine was observed and it is assumed that any cysteine formed during hydrolysis was oxidized to cystine during the preparatory processes (Stein & Moore, 1949).

When tyrosine and tryptophan both occur, then, at present, some other method should be used, e.g. one of the available colorimetric procedures.

The determination of cystine has not been worked out, but as it runs behind glycine in both solvents, it does not interfere with the above scheme. This amino acid, together with cysteine, is discussed below.

RESULTS

Made-up mixtures

The method has been applied to mixtures containing various amounts of some or all of the amino acids listed in Table 3. Two analyses of such a mixture are set out in Table 8, together with a summary of the recoveries experienced in the course of analyses of several such mixtures.

Natural products

Egg albumin. A sample of egg albumin (crystallized) obtained from Armour Laboratories was used. The nitrogen content was 15·45% on a moisture-free basis. Two independent hydrolyses were carried

Summary of scheme of analysis for monoamino acids

Completely separated	Direct estimation after use of periodate	Estimation by difference after periodate oxidation	Estimation by difference
Alanine (*P*)	Glycine (*P* and *B*)	Serine (*P* and *B*)	Leucine
Isoleucine (*B*)	Hydroxyproline (*P*)	Threonine (*P*)	Methionine
Phenylalanine (*P*)			Tyrosine
Proline (*B*)			(if tryptophan absent)
Valine (*B*)			
Threonine (*P*)			
(if hydroxyproline absent)			

(*P*) Propanol:water (80:20 by vol.).
(*B*) *iso*Butanol:methyl ethyl ketone:water (70:50:30 by vol.).

Table 8. *Analysis of a made-up mixture of amino acids*

Amino acid	Taken (mg./ml. soln.)	0·5 ml. soln. analysed: Gas evolved (μl. at s.t.p.)	0·5 ml. soln. analysed: Recovery (%)	1·0 ml. soln. analysed: Gas evolved (μl. at s.t.p.)	1·0 ml. soln. analysed: Recovery (%)	Range of recoveries for amino acids (N > 0·08 mg.) observed with numerous made-up mixtures
Glycine	1·614	92·2*	98·7	192·0*	102·8	103–97
Serine	1·782	66·0*	97·5	135·5*	96·4	105–95
Threonine	1·236	102·8	97·9	218·5	103·3	105–95
Alanine	1·600	197·1	98·1	390·8	97·3	103–97
Proline	1·352	135·6	100·6	235·8	99·0	103–97
Tyrosine	0·596	41·6	115	70·1	96·9	108–92
Valine	2·372	226·3	97·4	181·5*	97·7	103–97
Methionine	1·228	75·5	94·6	66·5*	104·2	105–90
Phenylalanine	1·680	102·6	96·6	221·5	104·3	105–95
Leucine	1·520	133·2	100·6	280·9	106·2	106–97
Isoleucine	1·360	113·9	95·8	226·1	95·2	99–93
(Leucine + isoleucine)	2·880	247·1	98·4	507	101	103–97

* Gas evolution for 40% of eluate.

out and two analyses made of each hydrolysate (Table 9).

Threonine, in the absence of hydroxyproline, was determined by direct oxidation with chloramine-T. The 'before glycine' value includes that part of the chromatogram between glycine and the starting line and will include any cystine present. An arbitrary carbon dioxide yield of 95% has been assumed for this.

Insulin. A sample of ox insulin (containing zinc) obtained from Boots Pure Drug Co. Ltd. was used. The nitrogen content was 15·12% on a moisture-free basis. One hydrolysis and two amino acid determinations were made (Table 10). The large tyrosine content caused some precipitation to occur on concentration and this was removed. No tyrosine figure is noted, therefore, but in any case the determination of tyrosine is more accurate when carried out independently on an alkaline hydrolysate of protein.

Table 9. *Monoamino acid composition of egg albumin* (*values are* N *as* % *protein* N)

	1st hydrolysate		2nd hydrolysate			Microbiological assay values	Other values
	1st det.	2nd det.	1st det.	2nd det.	Average		
Glycine {Propanol	4·34	—	4·08	4·11	—	—	—
Glycine {*iso*Butanol	4·31	—	4·23	4·20	4·21	4·2[1]	3·85[7]
Serine* {Propanol	6·68 (7·46)	—	6·28 (7·02)	6·29 (7·03)	6·47	7·92[1], 7·02[2]	6·92[8]
Serine* {*iso*Butanol	6·63 (7·41)	—	6·56 (7·33)	6·36 (7·11)	(7·23)	—	—
Threonine*	3·01 (3·18)	—	3·34 (3·53)	3·22 (3·40)	3·19 (3·37)	3·46[1], 2·7[3]	3·01[8]
Hydroxyproline	0	0	0	0	0	—	—
Alanine	—	6·40	6·17	6·41	6·33	7·47[4]	6·69[9]
Proline	2·84	2·71	2·65	2·85	2·76	6·16[1]	2·5[10]
Tyrosine	1·56	—	1·71	1·52	1·60	1·74[5]	2·0[10]
Valine	5·48	5·72	5·56	5·56	5·58	5·31[3]	3·5[9]
Methionine†	2·41	1·84	2·45	1·98	2·29	2·44[3]	3·1[11]
Phenylalanine	3·89	4·34	—	3·73	3·99	4·25[3]	4·0[9]
Isoleucine	4·31	4·50	4·30	4·22	4·33	4·74[3]	10·8[9]
Leucine	6·03	—	5·80	6·38	6·07	6·2[3], 6·6[6]	
'Before glycine'†	0·6	—	0·8	—	0·7	—	—
Total N of monoamino acids	47·5 (micro-Kjeldahl)		47·4 (micro-Kjeldahl)		47·6		
Insol. humin	7·5		7·2				
Ammonia (formed on hydrolysis)	9·0		8·7				

* Figures in brackets are values corrected for destruction during hydrolysis (Rees, 1946).

† See text.

(1), Sauberlich, Pearce & Bauman (1948); (2), Boyd & Logan (1942); (3), Stokes, Gunness, Dwyer & Caswell (1945); (4), Sauberlich & Bauman (1949); (5), Gunness, Dwyer & Stokes (1946); (6), Ryan & Brand (1944); (7), Stein (1943); (8), Rees (1946); (9), Tristram (1946); (10), Chibnall (1946); (11), Kassell & Brand (1938).

Table 10. *Monoamino acid composition of ox insulin* (*values are* N *as* % *protein* N)

	1st det.	2nd det.	Average	No. of residues/molecule: Found*	Reported†
Glycine	6·38	6·30	6·34	8·4	8
Serine‡	4·01 (4·42)	4·27 (4·77)	(4·63)	6·1	6
Threonine‡	2·21 (2·34)	2·24 (2·37)	(2·36)	3·1	2
Hydroxyproline	Absent		0	0	0
Alanine	4·84	4·97	4·91	6·5	6
Proline	1·55	1·64	1·60	2·1	2
Valine	7·15	7·14	7·08	9·5	10
Valine	6·96§	—	—	—	—
Methionine	Absent		0	0	0
Phenylalanine	3·91	3·99	3·95	5·2	6
Isoleucine	1·40	10·1	1·40	1·9	2
Leucine	8·60		8·60	11·4	12
'Before glycine'	5·0	—	5·0	—	—

* Calculated assuming mol.wt. 12000 and N content 15·54% for Zn-free insulin.

† Compiled from Sanger & Tuppy (1951) and Sanger & Thompson (1953).

‡ Bracketed values are corrected for hydrolysis losses (Rees, 1946).

§ Determined on propanol:water chromatogram in absence of methionine.

DISCUSSION

The essential difference between the chloramine-T reaction as performed by Krebs (Cohen, 1940) and Van Slyke *et al.* (1941) is that Krebs, using a Warburg respirometer, was measuring total gas evolved, whereas Van Slyke *et al.*, by first absorbing the carbon dioxide in alkali, were ensuring that only carbon dioxide was measured. A pointer to the reason for Krebs's high recoveries is contained in a footnote to Cohen's paper, where it is stated that ammonium salts interfere with the estimation, giving gaseous nitrogen with chloramine-T. While Dakin (1916) showed that excess of chloramine-T gives a nitrile instead of aldehyde and ammonia, in no case were his yields quantitative. A simple calculation from the data of Table 4 shows that if, at pH 4·7, 10 % of the nitrogen of an amino acid should form ammonia instead of nitrile, then this would result in an increase of approximately 10 % in the apparent amount of carbon dioxide evolved, and it is significant that glycine, which gives only a trace of hydrocyanic acid (Dakin, 1916), should give the greatest evolution of gas (Table 1).

It is suggested, therefore, that the high results obtained by Krebs are due to the formation in part of ammonia which is then oxidized to gaseous nitrogen. This suggestion is given added weight by the observed effect of formaldehyde on the evolution of gas both from ammonia and from amino acids. At pH 2·5 the amount of gas obtained from ammonia is very greatly diminished by the presence of formaldehyde (Table 4), while the amount of gas evolved by the individual amino acids is also decreased (Table 1).

Ammonia is known to combine with formaldehyde in a variety of ways, but it is surprising to find that this combination is apparently much stronger at pH 2·5 than at pH 4·7.

When any method of analysis is based on a factor representing other than theoretical recovery, it is essential to show that this factor does in fact remain constant during the differing conditions that may be experienced. The conditions inside the Warburg flasks, however, are quite rigidly defined and the only variables are the concentration of amino acid and the concentrations of other amino acids also present by reason of the limitations of the chromatographic separation.

Determinations carried out over a wide range of concentration did not show any variation in percentage carbon dioxide recoveries, except in the case of serine and of threonine (Table 2) where the variations are not large; furthermore, serine and glycine are completely indifferent to each other's presence in their reaction with chloramine-T.

The method was applied to egg albumin and insulin hydrolysates; no difficulties arose except on account of the very high tyrosine of insulin, which has been mentioned above. There would be nothing to prevent the accurate determination of tyrosine in other materials containing a moderate proportion of this amino acid; this has been confirmed in this laboratory in work on plant extracts.

It is of interest to compare the protein results with those obtained by other workers. For egg albumin, the list of references is by no means exhaustive, but there is generally good agreement with results either from microbiological assay or by chemical methods, although it must be admitted that the range of published figures is often disconcerting. The disparity between the first and second determinations of methionine is probably due to the ease with which this amino acid is oxidized, the diminution being a measure of the extent of oxidation rather than experimental error. Stein & Moore (1948) have stabilized methionine by the inclusion of 2:2′-thiodiethanol (thiodiglycol) in their solvent. Comparison of insulin figures has been with those implied by partial hydrolysis studies (Sanger & Tuppy, 1951; Sanger & Thompson, 1953) (Table 10). Agreement is excellent except for the threonine value.

It is felt that the method described may well be capable of further refinement. The use of longer papers, or the elaboration of a third solvent system to enable tyrosine and methionine to be determined directly might well be advantageous. Nevertheless, the method as described would appear to be relatively simple and of an accuracy great enough to allow its application to many biochemical problems.

SUMMARY

1. Paper partition chromatography has been applied to the separation of the neutral amino acids isolated by ionophoresis. The method has particular value for the free amino acids to be found in plant extracts.

2. Determination of the separated amino acids eluted from the chromatograms depends on the evolution of carbon dioxide in a Warburg respirometer following oxidation with chloramine-T. The following degree of accuracy has been achieved in the analysis of made-up mixtures of amino acids within the optimum range for each amino acid of 0·08–0·25 mg. N: leucine + isoleucine, valine proline, analine and glycine (10 ± 03 %); isoleucine (99–93 %); leucine (106–97 %); threonine (in absence of hydroxyproline), serine, phenylalanine (100 ± 5 %); tyrosine (in absence of tryptophan) (100 ± 8 %); methionine (105–90 %).

3. The method has been applied to hydrolysates of egg albumin and insulin, and the results compared with those obtained by other analytical techniques.

4. It is suggested that, because of its relative simplicity, the method is suitable for application to routine procedures.

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The Carotenoids of the Carapace of the Echinoderm *Ophidiaster ophidianus*

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In 1934 Karrer & Benz isolated astacin from the bright pink shells of *Ophidiaster ophidianus*; it is now known (Kuhn & Sörensen, 1938) that astaxanthin is the naturally occurring pigment and that astacin is a manipulative artifact. Karrer & Benz did not report the presence of any other carotenoid. As the importance of the presence of trace carotenoids in understanding the metabolism of carotenoids is increasing, it was desirable to find out if *Ophidiaster ophidianus* accumulated carotenoids other than astaxanthin.

EXPERIMENTAL

Material. The material was collected in Galli of Positano (Naples), and two groups of fourteen animals were examined. The animals were all about 14 cm. in diameter.

Extraction of material. The animals were opened and the internal organs removed completely and discarded. The clean shells were covered with acetone and allowed to stand for 5 min. at room temperature. The acetone was then removed by decantation and the carapaces were re-extracted with further quantities of acetone (usually 3–4 times) until all the pigments were removed. The combined acetone extracts were concentrated at room temperature at the pump, diluted with an equal volume of water and the pigment was extracted with diethyl ether. The ether extract was dried over anhydrous Na_2SO_4 and the solvent then removed under N_2. The residue was redissolved in light petroleum (b.p. 35–50°) and subjected to chromatographic analysis. When it was not possible to examine the extracts immediately, they were stored below $-20°$ in an ice-box.

Chromatographic analysis. Six separate chromatographic analyses were carried out (on portions of the two extracts). The results were identical in each case. The separations were carried out as follows:

(1) A preliminary separation of the pigment mixture was carried out on $CaCO_3$ (British Drug Houses Ltd., A.R. grade), using light petroleum (b.p. 35–50°) containing 4% (v/v) of ethanol as developer. The resulting chromatogram is described in Table 1.

(2) Fraction 1*A* was rechromatographed on alumina (Merck-Brockmann) deactivated by suspending it in methanol for about 30 min., filtering off the methanol and drying overnight at room temperature (Goodwin, 1952*a*). Light petroleum, containing different concentrations of diethyl ether, was used as developer. Eight fractions were obtained as recorded in Table 2. Fractions 2*A* and 2*B* were separated using pure light petroleum and passed straight through the column. On development of the column with light petroleum containing 1·5% (v/v) of diethyl ether,

Table 1. *The first separation of* Ophidiaster *carotenoids*

Adsorbent, $CaCO_3$; developer, light petroleum containing 4% (v/v) ethanol; the fractions are in order of increasing adsorptive power.

Fraction no.	Description	Absorption spectrum maxima in CS_2 (mμ.)
1*A*	Orange-red; passes straight through the column; a mixture (see Table 2)	—
1*B*	Reddish pink	505
1*C*	Orange-red	500
1*D*	Yellow; only present in traces; not examined further	—

fractions 2*C* and 2*D* separated and moved off the column. Fractions 2*E* and 2*F* also separated but were not eluted until the concentration of ether was increased to 12 and 18% (v/v), respectively. The residual, red pigment separated into three fractions (2*G*, 2*H* and 2*I*) when the concentration of ether was further increased. The most strongly adsorbed (2*I*) was eluted only with ethanol.

Quantitative measurements. The various fractions were collected and made up in known volumes of light petroleum and the extinction of the solutions measured at the wavelength of maximal absorption of the pigment concerned. The amount of each pigment present could then be calculated using the $E^{1\%}_{1\,cm.}$ ($\lambda_{max.}$) values recorded by Karrer & Jucker (1949) and Goodwin (1952*b*). In the case of the unidentified carotenoids, $E^{1\%}_{1\,cm.}$ ($\lambda_{max.}$) was assumed to be 2500.

RESULTS

Identification of the pigments

Fraction 1*B* (the major one) is undoubtedly unesterified astaxanthin, as observed by Karrer & Benz (1934). It is hypophasic when partitioned between light petroleum and 90% (v/v) aqueous methanol and exhibits a single, banded absorption spectrum with its maximum at 470 mμ. in light petroleum and at 505 mμ. in CS_2.

Fraction 1*C* is astacin; it is more strongly adsorbed than astaxanthin and has the same shaped curve but with its maximum shifted to slightly lower wavelength (500 mμ. in CS_2) (Goodwin & Srisukh, 1949). It is probably an artifact produced during the extraction procedures.

Fraction 2*B* was proved to be β-carotene by the identity of its absorption spectrum with that of pure β-carotene and the fact that when mixed with an authentic specimen and chromatographed on strong alumina, no separation could be obtained.

Fraction 2*E* is cryptoxanthin. Only two pigments have absorption spectra very similar to that of β-carotene, cryptoxanthin and zeaxanthin. In the phase test with 90% (v/v) aqueous methanol, the former is epiphasic and the latter hypophasic (Karrer & Jucker, 1949). Fraction 2*E* is also epiphasic and, furthermore, is equally distributed between the two phases when partitioned between light petroleum and 95% (v/v) aqueous methanol, a further characteristic test for cryptoxanthin. Finally, a comparison of the adsorptive power of 2*E* with that of cryptoxanthin and zeaxanthin (Goodwin, 1952*b*) shows 2*E* to be the same as that of cryptoxanthin.

From its position on the column (Table 2) and its absorption spectrum, fraction 2*A* appears to be a *cis*-isomer of β-carotene, probably *neo*-β-carotene B (Zechmeister, 1944). α-Carotene, which would have about the same adsorptive power, appears to be ruled out because of the lack of definition of the absorption spectrum of 2*A* (Fig. 1).

Fig. 1 also illustrates the absorption spectrum of 2*C*, the maxima of which are characteristic of γ-carotene; its position on the column is also characteristic of γ-carotene (Goodwin, 1952*a*). Because of the very small amounts of this pigment which are in *Ophidiaster*, it has not been possible to confirm completely its identity with γ-carotene; the slight doubt which remains is due to the fact that it has never been possible to get a specimen of 2*C* with the same shape of absorption curve as γ-carotene.

Table 2. *Chromatographic separation of fraction* 1*A of Table* 1

Adsorbent, deactivated alumina; developer, light petroleum containing diethyl ether; the fractions are in order of increasing adsorptive power. Figures in brackets denote inflexions.

Fraction no.	Description	Absorption spectrum maxima in light petroleum (mμ.)	Ether required to separate pigment from that named above (%, v/v)
2*A*	Yellow, diffuse	(420), 442, 472	0
2*B*	Orange, diffuse	(420), 448, 476	0
2*C*	Rose	(430), 460, 485	1·5
2*D*	Yellow	448	1·5
2*E*	Orange-yellow	(420), 448, 475	12
2*F*	Yellow	455	18
2*G*	Red-pink	460	24
2*H*	Red-pink	470	50
2*I*	Red-pink	470	Ethanol

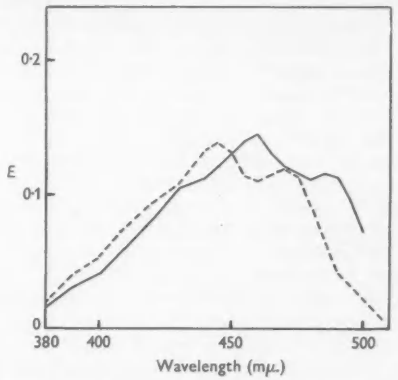


Fig. 1. Absorption spectrum of pigments. 2*A*, - - - -; and 2*C*, ——. Solvent, light petroleum (b.p. 35–50°).

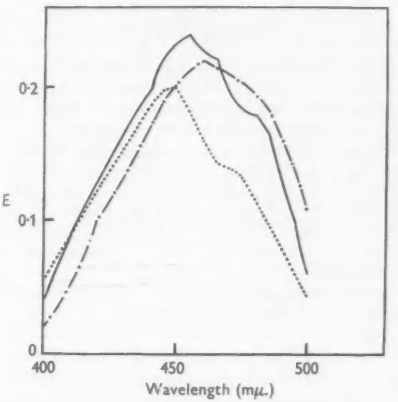


Fig. 2. Absorption spectra of pigments. 2*D*, ...; 2*F*, ——; and 2*G*, ·—·—· Solvent, light petroleum (b.p. 35–50°).

Fraction 2*D* (Fig. 2) is not identifiable with any known pigment; its spectrum suggests the presence of a keto group, but its weak adsorptive power seems to preclude this. A very similar pigment is widely distributed in berries (Goodwin, 1953*a*).

Fractions 2*F* and 2*G* appear to be new pigments for a survey of the literature (Goodwin, 1952*c*) did not reveal any carotenoid with the properties of these two pigments. From the fact that both have essentially single-banded spectra (Fig. 2) they are probably keto-carotenoids. Fractions 2*H* and 2*I* are traces of astaxanthin and astacin, respectively, carried through during the first fractionation.

Quantitative experiments

Four experiments were carried out to determine the relative distribution of the various pigments in *O. ophidianus*; the mean results are given in Table 3. It will be seen that in agreement with the findings of Karrer & Benz (1934), astaxanthin is the major component.

Table 3. *The quantitative distribution of carotenoids in the carapaces of* Ophidiaster ophidianus

(*neo*-β-Carotene B and γ-carotene have not been unequivocally identified. The traces of astacin produced during manipulation have been ignored.)

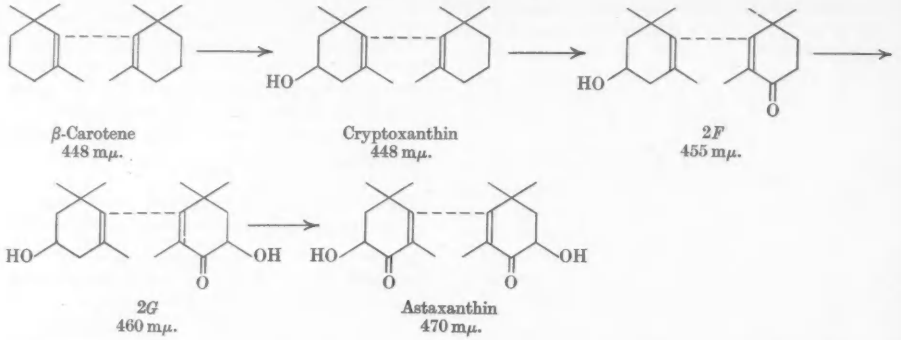
Carotenoid	Amount (% of total pigments listed)
neo-β-Carotene B	6·0
β-Carotene	6·8
γ-Carotene	0·8
2*D*	3·3
Cryptoxanthin	11·0
2*F*	4·8
2*G*	10·0
Astaxanthin	57·3

DISCUSSION

This investigation shows the importance of re-examining animal tissues for carotenoids in the light of modern information. Although astaxanthin, the only pigment identified by Karrer & Benz (1934), is by far the major component, there are small but important amounts of seven other pigments. Of special interest are two, 2*F* and 2*G*, which appear to be entirely new. Not only are these new, but tentative structures can be assigned to them which would suggest that they might be intermediates in the synthesis of astaxanthin from β-carotene.

Considering the absorption spectra maxima of β-carotene and astaxanthin, introduction of two 3-hydroxy-4-keto groupings results in a shift in wavelength of 22 mμ., i.e. from 448 to >470 mμ. The introduction of one such grouping would result in a shift of 11 mμ., i.e. about 5 mμ. more than if the substitution were a simple 4-keto substitution, e.g. echinenone, 4-oxo-β-carotene, $\lambda_{max.}$ 452 mμ. (Goodwin, 1953*b*). The wavelength (458–460 mμ.) given by Goodwin & Taha (1950) is too high.

Fraction 2*F*, which has a very similar spectrum to that of echinenone, but which is more strongly adsorbed, might be 3-hydroxy-4′-oxo-β-carotene (455 mμ.); addition of a hydroxy group at 3′ would shift this band to about 460 mμ. (2*G*), and the introduction of a keto group at position 4 (i.e. next to an isolated —OH group) would increase λ_{max} by 10 to that of astaxanthin. Thus a possible scheme of synthesis of astaxanthin from β-carotene could be represented as shown on p. 558.



β-Carotene 448 mμ. | Cryptoxanthin 448 mμ. | 2F 455 mμ.

2G 460 mμ. | Astaxanthin 470 mμ.

Much further work is required before this scheme can be fully established or otherwise, but it is important to point out that β-carotene has been shown to be almost certainly a precursor of astaxanthin in locust eggs (Goodwin & Srisukh, 1949).

This work on *O. ophidianus* emphasizes the importance of the study of the minor carotenoid components in living tissues.

SUMMARY

1. The presence of astaxanthin in the shell of *Ophidiaster ophidianus* is confirmed, and that of β-carotene and cryptoxanthin demonstrated. *neo*-β-Carotene B and γ-carotene are also probably present.

2. Three new pigments are reported: two are probably keto-carotenoids.

3. The quantitative distribution of carotenoids in the shell of *O. ophidianus* is recorded.

4. A possible biosynthetic route from β-carotene to astaxanthin is discussed.

I wish to express my deep gratitude to Dr T. W. Goodwin for reading the manuscript and for helpful advice and criticism.

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The Metabolism of Collagen and other Proteins of the Skin of Rabbits

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It has been shown with the aid of [α-^{14}C]glycine that, in the adult rat, collagen from a wide variety of sources is metabolically almost inert, if compared with proteins such as those of plasma or of liver (Neuberger, Perrone & Slack, 1951; Neuberger & Slack, 1953). Results indicating a relatively slow turnover of collagen in guinea pigs (Robertson, 1952) have also been reported. However, in the growing rat, labelled glycine is incorporated into collagen at a fairly fast rate (Neuberger *et al.* 1951) and the use of this amino acid thus seemed to afford a convenient tool to examine the mechanism by which this fibrous protein is formed.

It was reasonable to assume that insoluble collagen is derived from a more soluble precursor and the presence of soluble, collagen-like proteins in connective tissue supports such a hypothesis. It was first observed by Zachariades (1900) that the tendon of the rat tail, if suspended in very dilute formic acid or acetic acid, swelled markedly and

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then began to dissolve. This dissolution of collagen, particularly that of the tail tendon of the rat, was studied in great detail much later by other French workers, particularly Nageotte, Guyon and Fauré-Fremiet and also by Leplat. Nageotte (1927*a*, *b*) found that rat-tail tendon partly dissolved if placed in solution of about 8×10^{-3}M acetic acid; the dissolution was not complete, since after three extractions about 70% of the tendon remained undissolved (Nageotte & Guyon, 1934). Nageotte (1927*b*, 1928) at first believed that this phenomenon was peculiar to the tail tendon of the rat, but Leplat (1933*a*, *b*) showed that the presence of soluble collagen could be demonstrated in other tissues of the rat and also in other species; this observation was confirmed by Nageotte (1933). The solutions of collagen thus obtained, although extremely dilute, were very viscous (Nageotte, 1927*d*) and showed marked laevorotation (Fauré-Fremiet, 1933*a*); on heating to about 60° an apparently irreversible change occurred, akin to denaturation, and gelatin was produced (Nageotte & Guyon, 1933). Nageotte's results with rat-tail tendon were confirmed by Huzella (1932) and also by Fauré-Fremiet (1933*a*, *b*) who treated the tendon with trypsin and lime water, before exposing it to dilute acetic acid. It is of considerable physiological interest that the proportion of collagen which is soluble in dilute acid was found to decrease with age (Nageotte & Guyon, 1934). This acid-soluble collagen has been named collagen A by Nageotte (1930), while the term collagen B has been applied to material made soluble in acid by preliminary treatment with alkali.

On dialysing the collagen solution against distilled water a gel was formed (Nageotte, 1927*d*), if the pH was above 5·0 (Guyon, 1934): at a lower pH no visible precipitation occurred. Addition of sodium chloride in high concentration produced a massive clot, but at low ionic strength fibrils were formed, the size and shape of which appeared to vary with the salt concentration and probably also with pH (Nageotte, 1927*a*–*d*). Some of the fibrils had a crystalline appearance, resembling tyrosine crystals and were birefringent (Nageotte, 1927*a*, *b*; Fauré-Fremiet, 1933*a*, *b*). The reconstituted fibrils closely resembled native collagen fibres as observed in sections of connective tissue and their staining characteristics were those of collagen. Fauré-Fremiet (1933*a*) also observed that soluble collagen was precipitated by chondroitin sulphate. The X-ray diagrams of reconstituted collagen fibres were found to be closely similar to those of native mammalian collagen (Wyckoff & Corey, 1936; Champetier & Fauré-Fremiet, 1938) and the same applied to their electron micrographs (Schmitt, Hall & Jakus, 1942).

More recently, Russian workers, especially Orekhovich and his co-workers, have studied the properties and distribution of soluble collagen in a great variety of species and tissues. Their method of isolation consisted of a preliminary extraction of the macerated tissue with a disodium hydrogen phosphate solution, which removed proteins of albumin and globulin type; this was followed by a further extraction with an acidic buffer such as citrate or oxalate (Tustanovskii, 1947; Orekhovich, Tustanovskii, Orekhovich & Plotnikova, 1948). Dialysis against a solution of low ionic strength and of a pH near neutrality gave needles of crystalline appearance of similar type, but of apparently more regular shape, than those described by Nageotte & Fauré-Fremiet. The material studied by Orekhovich *et al.* (1948) appears to be identical with, or closely similar to, that described by the French workers. Orekhovich called this soluble protein 'procollagen', implying that it is a precursor of insoluble collagen. The introduction of the term 'procollagen' has been deplored by Bear (1952), and it may be noted that the term 'précollagène' has been used by Laguesse (1903) to describe amorphous material present in connective tissues, lying between collagen fibres, and having some of the staining characteristics of collagen. The investigations of the Russian workers which have been summarized by Orekhovich (1952) show that the amino-acid composition of the soluble collagen is on the whole similar to that of insoluble collagen, although definite differences were observed. The ultraviolet absorption spectrum resembled that of gelatin. Bresler, Finogenov & Frenkel (1950) have studied the behaviour of 'procollagen' solutions in the ultracentrifuge and their results suggest that the molecules are rod shaped, and Orekhovich (1952) has reported on the electrophoretic behaviour of this protein. It has also been shown that soluble collagens occur in a great variety of tissues in all of the many vertebrates examined (Plotnikova, 1947). Earlier findings which suggested that the soluble material occurs in relatively large amounts only in connective tissue of young animals were extended and confirmed by Orekhovich (1950).

The presence of possibly another type of soluble collagen is suggested by the work of Highberger, Gross & Schmitt (1951), who found that an extract of skin made with slightly alkaline phosphate solutions contained a protein which in its appearance in the electron microscope resembled collagen.

The present work was carried out with young rabbits and was intended to answer the question whether soluble collagens could, as judged by their capacity to incorporate labelled glycine, be precursors of insoluble collagen. Some of our results have already been briefly reported (Harkness & Neuberger, 1952; Harkness, Marko, Muir & Neuberger, 1953), and observations similar to those contained in our first preliminary communication

have also been mentioned by Orekhovich (1952). In connexion with the isotope work a preliminary survey of proteins in rabbit skin was undertaken.

EXPERIMENTAL

Treatment of animals. Male and female albino rabbits of the Copenhagen or Himalayan strain were used throughout. Their weights at the time when the labelled glycine was given varied between 500 and 750 g. and they were about 4 weeks old. In some experiments litter mates were used. The animals were fed diet no. 18 (Bruce & Parkes, 1946) *ad lib.*

In the first series of experiments the [α-^{14}C]glycine of specific activity of 1·7 or 5·2 μc/mg. was given intraperitoneally in 0·5–1·0 ml. of 0·9% NaCl; the dose injected was 10 μc/100 g. body wt. In the second series of experiments the [α-^{14}C]glycine of specific activity 1 mc/19·3 mg. was given by mouth; rabbits were starved for the 24 hr. period preceding the feeding of the labelled glycine, but were allowed free access to water; total activity fed was 20 μc/100 g. body wt. The glycine was dissolved in a small amount of water and about 4 g. of pellets (diet no. 18) were impregnated with the glycine solution. The pellets after being dried were fed to the starved rabbits; they were generally eaten within 1 hr.

In the first series of experiments the rabbits were killed by breaking the neck. In later experiments, in which the radioactivity of the glycine in plasma proteins was examined, the animals were anaesthetized by injection of pentobarbitone followed by inhalation of chloroform, the thorax was opened and the heart exposed. About 20–30 ml. of blood were then removed by heart puncture, using a heparinized syringe.

Plasma proteins. The blood obtained by heart puncture was centrifuged to remove red and white cells. The plasma, to which was added non-radioactive glycine to a final concentration of 0·5%, was dialysed against frequent changes of distilled water.

Isolation of skin proteins

The general procedure is given below; any important deviations will be referred to under Results.

The hair was removed either with the aid of an electric clipper or by shaving and the skin and hypodermis were stripped off the body; the skin covering the paws, tail, ears and tip of nose was not used. The hypodermis, consisting of muscle, fat and connective tissue, was then scraped off. The remaining skin, consisting of dermis, epidermis and hair roots and stumps was then rolled up and frozen in solid CO_2, cut into small pieces with a scalpel and finely ground. Between 30 and 50 g. of skin were obtained from a rabbit. All further operations were carried out at 2° using 2-octanol as preservative. Extractions were done either on a mechanical shaker or with the aid of stirring; the solvents used were similar to those of Orekhovich *et al.* (1948). The main steps in the fractionation are shown in Fig. 1.

Proteins extracted by disodium hydrogen phosphate. The macerated skin was suspended in about 10 times its weight of a solution which had been saturated with Na_2HPO_4 at 2°. The extraction was continued for 8–20 hr., and the mixture was then centrifuged for 0·5 hr. at 2500 rev./min. The supernatant fluid was decanted and in most experiments filtered through a fine sintered-glass funnel (H3). The residue was re-extracted with the saturated phosphate solution up to six times until the last extract was water-clear. The extracts were centrifuged and filtered as described.

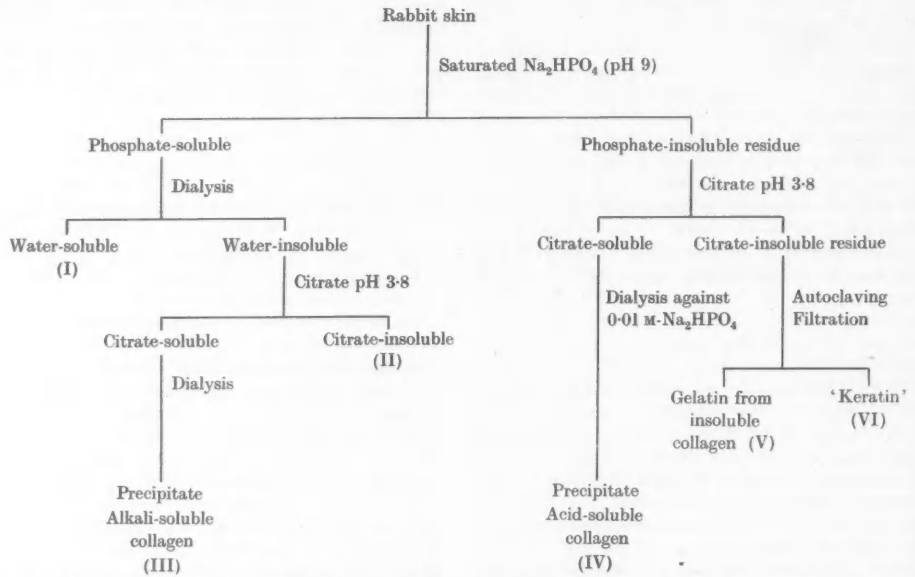

Fig. 1. Scheme of fractionation of skin proteins of young rabbits. All operations were carried out at 2°.

The combined phosphate extracts were dialysed for 36–48 hr. against distilled water. The precipitate which had formed was removed by centrifuging (1 hr. at 2500 rev./min.). The clear supernatant was concentrated to dryness and the residue designated 'water-soluble' fraction (Fig. 1, fraction I).

To the solids obtained from the dialysis against water were added 100 ml. 0·1 M sodium citrate/NaCl buffer, pH 3·8 (Sørensen, 1912). The suspension was shaken for 8–10 hr. and centrifugation and filtration through a sintered-glass filter or paper were done as described above. The extraction was repeated three or four times. The residue remaining after these extractions was designated the 'citrate-insoluble' fraction (Fig. 1, fraction II).

To the combined extracts which contained material, soluble in the citrate buffer but insoluble in water, was added non-radioactive glycine (final concentration 0·5%); the solution was dialysed first against running tap water and then against frequent changes of distilled water. After prolonged dialysis a fine fibrous precipitate appeared which was collected by centrifugation. It was named 'alkali-soluble' collagen (Fig. 1, fraction III), since it was originally soluble in phosphate buffer, pH 9. However, the solid material, obtained after the operations described, could not be redissolved in a neutral or alkaline buffer. The weight of this fraction varied between 10 and 25 mg. for one rabbit skin. The protein was dissolved in 5–10 ml. 0·1% (v/v) acetic acid, and the solution heated to 80–90° for 10 min. The solution, while hot, was filtered under slight pressure, through a fine sintered-glass funnel (H 3). The filtrate was usually faintly opalescent.

Proteins soluble in citrate buffer, pH 3·8, *but insoluble in phosphate solution*, pH 9. To the residue remaining after thorough extraction with saturated Na_2HPO_4 solution was added citrate buffer, pH 3·8, in amounts corresponding to 10 times the weight of original skin. Extraction was carried out with stirring or shaking for about 18 hr.; when centrifuged and filtered, the extract gave a slightly viscous, but water-clear, solution. The solid was re-extracted in a similar manner 3 or 4 times. In some experiments, to be described later, the extracts were worked up separately, whilst in others they were combined. The solutions were dialysed against large volumes of 0·01 M-Na_2HPO_4. A heavy fibrous precipitate soon appeared; this solid was collected by centrifuging at 2500 rev./min. for 0·5 hr. In most isotope experiments the precipitate was redissolved in a small volume of citrate buffer and non-radioactive glycine was added to a final concentration of 0·5%. The solution was dialysed first against several changes of the citrate buffer and then against 0·01 M-Na_2HPO_4. The precipitate was collected as before, washed three times on the centrifuge with 100 ml. 10% (w/v) NaCl and finally with distilled water. Repeated washing with water converted the fibrous precipitate into a thixotropic gel. The precipitate of 'acid-soluble collagen' (Fig. 1, fraction IV) was generally left in contact with water for no longer than 1 hr. Conversion into gelatin was done by heating in water to 60° for 1 hr. or 80–90° for 15 min. The gelatin solution was filtered through a sintered-glass funnel under slight pressure. In some experiments the gelatin solution was poured into 10 vol. of acetone and the precipitate allowed to stand at 0° for 18 hr. It was then centrifuged, washed successively with ethanol:ether (3:1; v/v) and ether and dried over P_2O_5 *in vacuo*.

Insoluble collagen (Fig. 1, fraction V). The residue remaining after extraction with citrate was washed on the centrifuge three times with 10% (w/v) NaCl (500 ml. portions) and then three times with water (500 ml. portions). In earlier experiments the washings were carried out over periods of days or weeks at 0°, but this did not apparently affect the properties of the final product. The material which had swollen with water treatment was autoclaved at a pressure of 15 lb./sq.in. for 3 hr. in the presence of 500 ml. water. The hot mixture was then filtered by gravity and an insoluble residue (Fig. 1, fraction VI) was obtained, consisting presumably of keratin, elastin and proteins denatured during the various operations. The filtrate containing the gelatin was concentrated to about 50 ml. and any solid which appeared was removed by filtration. The gelatin was either precipitated with acetone as described above or concentrated to dryness and redissolved in about 50 ml. of water, filtered through a fine sintered-glass funnel (H 3) and stored. The term 'insoluble collagen' used hereafter refers to this fraction.

Isolation of 2:4-dinitrophenylglycine (DNP-glycine) and radioactivity measurements

The various protein fractions were hydrolysed by heating with 6 N-HCl for 48 hr. in sealed tubes in an oven kept at 105°. The hydrolysates were concentrated to dryness *in vacuo* and the residues taken up in water; humin was removed by filtration before analyses were carried out. The preparation and isolation of DNP-glycine was generally done by the method of Perrone (1951) as described by Neuberger *et al.* (1951). In a few experiments in which the amount of material was small, the method of Campbell & Work (1952) was used for the isolation of DNP-glycine. Radioactivity measurements were made as described by Neuberger *et al.* (1951). Results are expressed as counts/min./sq.cm. (at 'infinite thickness').

Chemical analyses

Total nitrogen. In most experiments this was determined according to Chibnall, Rees & Williams (1943). For the fractionation experiments described in Table 1 the method of Ma & Zuazaga (1942) was used.

Tyrosine. This was determined according to Udenfriend & Cooper (1952). Readings were made on a Unicam Spectrophotometer at 450 mμ.

Hydroxyproline. The method of Neuman & Logan (1950) was used. Readings were made on a Unicam Spectrophotometer using a wavelength of 550 mμ.

Glycine. This amino acid was determined by the method of Krõl (1952). Readings were made on a Hilger Spekker Photometer using a violet spectrum filter (Ilford no. 601; 400–450 mμ.).

RESULTS

Characterization of the proteins of rabbit skin

The protein fractions obtained from the combined skins of two young rabbits by the methods outlined in Fig. 1 were analysed for total nitrogen and hydroxyproline, tyrosine and glycine contents (Table 1). It will be observed that only 75–80% of the total nitrogen, hydroxyproline and tyrosine of the skin are recovered in the various fractions listed in Table 1. This is due to the loss, which was not determined, of low-molecular nitrogenous substances by diffusion during dialysis, and adsorption

Table 1. *Distribution of nitrogen and of some amino acids in the various fractions of rabbit skin*

Roman numerals refer to fractions in Fig. 1 and text. Figures in brackets are calculated values of hydroxyproline-N, tyrosine-N and glycine-N, respectively, as percentage of total N of fraction.

Fraction	Nitrogen (mg.)	Hydroxyproline (mg.)	Tyrosine (mg.)	Glycine (mg.)
Total skin	1792	776 (4·6)	256 (1·1)	1432 (14·9)
Total phosphate soluble*	278	28 (1·1)	48 (1·1)	87 (5·8)
Water-soluble (I)	95	Approx. 1 (0·1)	28 (2·3)	21 (4·1)
Citrate-insoluble (II)	47	5 (1·1)	8 (1·3)	7 (2·7)
Alkali-soluble collagen (III)	21	16 (8·4)	0·2 (0·1)	29 (25·8)
Acid-soluble collagen (IV)	123	102 (8·8)	6 (0·37)	170 (25·7)
Water washings of citrate-treated skin residue	68	45 (7·1)	2·8 (0·32)	61 (16·8)
Skin residue†	964	442 (4·9)	154 (1·2)	1029 (19·9)
Gelatin from insoluble collagen (V)	598	421 (7·5)	35 (0·45)	858 (26·7)
'Keratin' (VI)‡	366	21 (0·6)	119 (2·2)	171 (8·7)

* Analyses on the material soluble in a saturated Na_2HPO_4 solution at 2° at a pH about 9.
† The residue remaining after the extraction of the skin with the saturated phosphate solution, followed by the extraction with the citrate buffer, washing with 10% NaCl and water.
‡ Obtained by difference: 'keratin' = skin residue – insoluble collagen.

of proteins on filter paper and membranes and by other mechanical causes. In addition, the various washing fluids contained small amounts of nitrogen and of the three amino acids which were determined in some cases. Making allowances for these losses the following conclusions can be drawn. The proteins of the skin of young rabbits can be divided into three broad groups: (*a*) Non-collagenous proteins soluble in phosphate buffer, pH 9 (fractions I and II). This group accounts for about 15% of the total nitrogen of the skin. (*b*) Proteins of collagen type. These account altogether for about 55% of the total nitrogen and can be divided into three fractions: material extracted by disodium hydrogen phosphate (fraction III), representing about 4% of the total collagen and 2% of the skin nitrogen; material not extracted by disodium hydrogen phosphate, but extracted by citrate, pH 3·8, and subsequent washing with water (fraction IV), and accounting for about 20% of the total collagen and about 10% of the skin nitrogen; and lastly 'insoluble' collagen (fraction V), not extracted by any of these procedures and representing about 75% of the total collagen and 35–40% of the skin nitrogen. (*c*) Insoluble proteins other than collagen (fraction VI). These account for 20–25% of the total nitrogen of skin and have average contents of tyrosine of about 5%, of hydroxyproline of 1% and of glycine of 7·5% (assuming arbitrarily a nitrogen content of 16% for these proteins). The occurrence of hydroxyproline in this fraction is probably due to incomplete removal of collagen. The rest of this fraction consists probably of keratin-like material and of other, probably intracellular, proteins, either not extracted by the procedures used or denatured during the various operations.

Soluble, non-collagenous proteins

In order to obtain more information about this fraction, the following experiment was done.

Skin (45 g. wet wt.) obtained from one adult rabbit was extracted with Na_2HPO_4 as described. The milky extract (pH 8·35) was dialysed for 18 hr. against tap water and then for 2–3 days against three changes of distilled water; a precipitate formed, which was collected on the centrifuge. It could not be redissolved in the Na_2HPO_4 solution originally used for the extraction, nor in sodium phosphate buffer at pH 7·8 or 6·8.

The solution, after dialysis, was freeze-dried and yielded 1·375 g. of material, corresponding to about 3% of wet skin. The solid was extracted with sodium phosphate buffer, pH 7·8, $I = 0·2$. Only about 20% dissolved, and the residue had become insoluble in the Na_2HPO_4 solution used for extraction. The solution of the soluble protein material was concentrated by pressure dialysis against phosphate buffer, pH 7·8, until the concentration of protein was about 1%. Serum proteins of the rabbit were found to dissolve almost completely after freeze-drying. Electrophoresis in the Tiselius apparatus was done both with the soluble protein fraction described above and rabbit serum.

The patterns were similar (Fig. 2), both in the position of the various peaks and in their relative

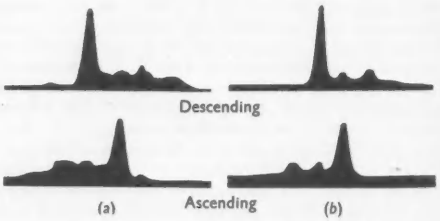

Fig. 2. Electrophoretic patterns of skin and plasma proteins of rabbit. (*a*) Pattern of skin protein fraction after 360 min.; buffer, potassium phosphate $I = 0·2$, pH 7·8; potential 2·9 v/cm.; protein concentration, approximately 1%. ($\Delta n_{25} = 0·00193$.) The skin protein is a soluble protein fraction of rabbit skin obtained on freeze-drying of a phosphate extract. (*b*) Pattern of plasma protein of a rabbit obtained under the same conditions as given above for the skin-protein fraction.

heights; but the skin sample also apparently contained a very small amount of material which migrated at pH 7·8 with a greater velocity than albumin.

Comparison of the three collagen fractions

Analyses for the hydroxyproline and tyrosine contents of the three collagen fractions are given in Table 2. The hydroxyproline values are of the same order as those found by other workers for mammalian collagens (Bowes & Kenten, 1948) and the tyrosine figures are all low. The variations between the values obtained for similar fractions in different experiments are somewhat larger than the experimental errors of the two methods, and it would appear that some of the differences shown in Table 2 between the three collagen fractions are significant.

Insoluble collagen has the lowest hydroxyproline and the highest tyrosine content; alkali-soluble collagen appears to have a slightly lower hydroxyproline content than acid-soluble collagen, but the tyrosine contents of the two fractions are similar and appreciably lower than that of insoluble collagen. From the data in Table 1 it can be calculated that the glycine content of all three samples is about the same, the glycine-nitrogen being 26–27 % of the total nitrogen.

The acid-soluble collagen fraction (IV) consisted mainly of needle-shaped 'crystals' similar to those described by Orekhovich *et al.* (1948) showing the typical banded structure of collagen in the electron microscope. There was usually also some amorphous material present. The solid alkali-soluble collagen (fraction III) consisted of a mass of fine fibres which showed a banded structure in the electron microscope. A two-dimensional paper chromatogram of an acid hydrolysate of this material showed a general picture similar to that obtained with a hydrolysate of the gelatin from insoluble collagen, or from acid-soluble collagen.

Table 2. *Hydroxyproline and tyrosine contents of the alkali-soluble, acid-soluble and insoluble collagen*

As percentage of total N of the hydrolysate.

Expt. no.	Hydroxyproline-N	Tyrosine-N
(*a*) Alkali-soluble collagen		
1	8·04	0·09
2	8·02	0·16
3	8·94	0·24
4	8·38	0·27
Av.	8·34	0·19
(*b*) Acid-soluble collagen		
1	8·79	0·37
2	8·77	0·21
3	9·55	0·34
4	8·79	0·29
Av.	8·98	0·30
(*c*) Insoluble collagen		
1	7·52	0·45
2	8·04*	0·47
3	7·02	0·62
4	7·74	0·49
Av.	7·58	0·51

* Gelatin solution refiltered to remove ppt. which had formed on standing at 0°.

For the interpretation of the isotope results it was important to assess the 'purity' of these fractions, i.e. to investigate whether these samples are free from non-collagenous proteins and also whether they represent chemically distinct groups. It has already been mentioned that the alkali-soluble collagen lost its solubility in the disodium hydrogen phosphate medium used for the original extraction, and the possibility existed that it was originally present in the form of fine fibres which passed through the filter used. Although this seemed unlikely, since filtration was done through a glass filter of low porosity, further evidence was sought. A phosphate extract of skin (25 ml.) was centrifuged at 10000 *g* for 1 hr. at 0°. A small amount of brown precipitate had formed and the supernatant was still faintly opalescent. Hydroxyproline estimation on measured samples indicated that the solution contained 0·518 mg. of this imino acid before centrifuging and 0·433 mg. after centrifuging. It would thus appear that the bulk of the alkali-soluble collagen was finely dispersed in the extraction fluid. The reason for the alteration of solubility of the alkali-soluble fraction during the isolation precedures is unknown, but may be due to a change akin to denaturation which occurred in spite of the low temperatures used. It is also possible that the material was kept in fine dispersion by other substances present in the original skin extract.

Acid-soluble collagen. If acid-soluble collagens were a group distinct from the insoluble collagens, it would be expected that, with repeated treatment of the skin residue with citrate, the amount of material removed by each successive extraction should decrease and ultimately approach zero. For the ten rabbits on which isotope data are given in Table 4, the total dry weight of acid-soluble collagen (fraction IV) was 17·6 ± 1·5 (S.E.M.) % of the total collagen (fractions IV and V), alkali-soluble collagen not being measured. Of the subfractions of the acid-soluble collagen, the mean weight of the first was 7·7, the second 4·6, the third 3·3 and the fourth 2·0 % of the total collagen. Extrapolation of the mean figures to an infinite number of extractions, suggests that the average proportion of acid-soluble collagen as percentage of total collagen would be about 20 %. However, it was observed that further extraction of the citrate-treated residue with water removed a further, relatively large amount of hydroxyproline-containing material (Table 1).

Although the amounts of collagen removed by successive extractions decreased progressively, there is not conclusive evidence that the change from acid-soluble to insoluble collagen is discontinuous.

The effectiveness of various solutions in dissolving acid-soluble collagen is shown in Table 3. The data were obtained by shaking portions of the damp fibres (fraction IV) with the various solutions at room temperature, and removing undissolved material on the centrifuge. There is no evidence that equilibration had taken place, but the relatively short period of extraction was used in order to reduce any irreversible changes which take place slowly at room temperature. The best pH for extraction appears to be near 3·0. With rising pH, extractability decreases markedly and becomes zero above pH 6·0. The slight effect of 0·1 N sodium hydroxide may be due to degradation. The slight effect of this solvent suggests that no significant amounts of acid-soluble collagen would be lost in the preliminary extraction used in the method of Lowry, Gilligan & Katersky (1941) for estimation of collagen. The effect of increasing concentration of calcium chloride in dissolving this collagen fraction is of interest. It has been noted already by Nageotte (1927*c*) that calcium chloride, unlike sodium or potassium chlorides, does not precipitate collagen which had been dissolved by acetic acid.

Table 3. *Extractability of acid-soluble collagen by different solvents*

The buffer covering the range of pH to 8·0 was that described by McIlvaine (1921), whilst that for the range pH 8·0–12·0 was that of Britton & Welford (1937). The molarities used were those given by the authors quoted. Extractability is given as mg. N/100 ml. solvent.

Solvent	Extractability
Citrate/phosphate, pH 2·5	30·2
Citrate/phosphate, pH 3·0	36·8
Citrate/phosphate, pH 3·5	24·6
Citrate/phosphate, pH 4·0	22·2
Citrate/phosphate, pH 4·5	6·2
Citrate/phosphate, pH 5·0	9·6
Citrate/phosphate, pH 6·0	3·1
Citrate/phosphate, pH 7·0	0·0
Citrate/phosphate, pH 8·0	0·0
Phosphate, pH 8·0	0·0
Phosphate, pH 10·0	0·0
Phosphate, pH 11·0	0·0
Phosphate, pH 12·0	0·0
0·3 M-Na_2HPO_4	0·0
0·1 M-NaOH	7·7
0·02 M-$Ca(OH)_2$	1·7
0·1 N-HCl	23·7
$1·6 \times 10^{-2}$ N acetic acid	48·7
$6·5 \times 10^{-4}$ N acetic acid	6·3
0·16 M-NaCl	1·4
0·8 M-NaCl	1·0
1·6 M-NaCl	1·0
0·13 M-KCl	1·1
0·65 M-KCl	2·3
1·3 M-KCl	0·7
0·09 M-$CaCl_2$	4·7
0·45 M-$CaCl_2$	12·6
0·9 M-$CaCl_2$	17·9

Insoluble collagen. Gelatin samples obtained by different methods and from different sources contain small, but varying, amounts of a substance which behaves in the different colorimetric tests like tyrosine. It has also been mentioned (see Table 2) that insoluble collagen, isolated as gelatin, contains more tyrosine than either of the two soluble collagen fractions. It thus appeared likely that the relatively high tyrosine content of the gelatin from insoluble collagen might be due to contamination by a non-collagenous protein. An attempt was therefore made to fractionate this gelatin. To such a gelatin solution at 0° was added trichloroacetic acid (TCA) to a final concentration of 5% (w/v); a precipitate was formed. The tyrosine content of the original gelatin was about 1%, whilst that of the material isolated from the TCA-supernatant was 0·5%. In another experiment in which gelatin, isolated from rabbits fed 24 hr. previously with [α-^{14}C]glycine, was used, the radioactivity of the DNP-glycine obtained from the TCA-supernatant was significantly less than the DNP-glycine of the untreated gelatin. Thus two samples from unfractionated gelatin gave values of 135 ± 1 and 131 ± 1 counts/min./sq.cm.; the corresponding samples from the TCA-supernatants were 97 ± 1 and 86 ± 1. Similar fractionation of gelatin from rabbits killed at later times after the administration of the isotope produced no significant change in the radioactivity of the isolated DNP-glycine.

Isotope experiments

In the first series of experiments, labelled glycine was injected intraperitoneally. With this route of administration surprisingly large variations were observed between the values obtained for similar protein fractions in different rabbits given identical doses of labelled glycine and killed after the same time intervals. Thus, two rabbits of similar body weight (nos. 3 and 7, Table 4) were given the same doses of [α-^{14}C]glycine 7 days before killing; it will be seen that radioactivities of all the fractions obtained from rabbit no. 3 are about 15 to 20 times higher than the corresponding values for rabbit no. 7. However, the ratios of radioactivities for different protein fractions obtained from the same rabbit showed a fairly regular behaviour; they were similar to those of other rabbits which had received identical treatment and depended almost entirely on the time interval between injection of labelled glycine and the killing of the animal. It has been our experience that similar relationships were found to apply to plasma and muscle proteins in rabbits which had been intraperitoneally injected with

Table 4. *Radioactivities of DNP-glycine samples prepared from skin proteins of rabbits which had received [α-^{14}C]glycine by intraperitoneal administration*

Fractions were not treated with unlabelled glycine.

Rabbit no. and sex	Interval between injection and killing (days)	Radioactivities of DNP-glycine (counts/min./sq.cm. at 'infinite thickness')						
		Total phosphate-soluble	Alkali-soluble collagen	Acid-soluble collagen				Insoluble collagen
				Extract 1	Extract 2	Extract 3	Extract 4	
A1 (M.)	1	28	—	4·8	2·6	2·8†		1·3
A5 (F.)	1	15·4	—	0	0	0 †		0
B1 (F.)	1	53*	169	9·5	3·6	2·4†		3·5
B2 (M.)	1	860*	1100	107	64	43 †		65
A2 (M.)	3	29·3	—	—	4·1	3·7†		2·4
A6 (F.)	3	490	—	370	284	263 †		134
A3 (M.)	7	194	—	270	330	306	303	186
A7 (F.)	7	12·3	—	21·2	20·5	15·3	13·0	8·8
A4 (M.)	21	34·4	—	36·1	55·6	62·0	55·1	65·6
A8 (F.)	21	8·7	—	12·5	13·0	14·5†		15·6

— Not isolated or estimated.

* These figures refer to the water-soluble fractions (I, Fig. 1) only. † Extracts 3 and 4 combined.

glycine; on intravenous or oral administration, radioactivities, even in rabbits, were much more reproducible. Rats which had received isotopic glycine by interperitoneal injection behaved in a fairly predictable manner (Arnstein & Neuberger, 1951). The reasons for these great variations of radioactivities after intraperitoneal injection in rabbits are obscure. It may be that in some cases the needle penetrated the caecum.

In most of the experiments of the first series glycine was isolated from the total phosphate-soluble protein fraction which included alkali-soluble collagen. On the other hand, the four fractions of acid-scluble collagen obtained from successive extractions with citrate were worked up separately. The phosphate-soluble proteins showed very high radioactivities relative to that of collagen 24 hr. after injection; after 3 days values for this fraction were still very much higher than the corresponding figures for insoluble collagen and appreciably higher than those found for the acid-soluble collagen fractions. The phosphate-soluble proteins had activities below those of any of the acid-soluble collagen fractions 7 days after injection and were similar to the values found for insoluble collagen, whilst after 21 days the radioactivities found for the phosphate-soluble fraction were lower even than those of the least active collagen.

The radioactivities of successive fractions of acid-soluble collagen relative to each other and relative to those of insoluble collagen appeared to show a fairly regular behaviour. With short intervals—1 and 3 days—radioactivities decreased sharply from the first to the fourth acid-soluble collagen fraction and then to insoluble collagen. With the 7-day samples insoluble collagen had still the lowest activity, but the differences between the various acid-soluble collagen fractions were small, whilst after 21 days insoluble collagen appeared to have the highest activity. These results are similar to those reported briefly by Orekhovich (1952) who determined the radioactivity of the whole protein.

In the second group of experiments (Table 5) glycine was fed by mouth over a very short period, the three fractions of the phosphate extract were worked up separately, but the acid-soluble collagen samples were combined. Plasma proteins were also prepared and from them glycine was isolated and its radioactivity assayed. In these experiments, as in the earlier series, insoluble collagen was less active than acid-soluble collagen 3 and 7 days after injection, but surprisingly this relationship was reversed in almost all the experiments in which the animals had been killed 8 or 24 hr., after injection of the labelled glycine. It has already been mentioned (p. 564) that the radioactivity of these insoluble collagen samples was reduced by treatment with TCA. The behaviour of alkali-soluble collagen was found to differ greatly from all other collagen fractions. At 8 hr. its activity was relatively low, but after 24 hr. the radioactivities of this fraction were of the same order as those found for plasma proteins, or citrate-insoluble or water-soluble skin proteins (Fig. 3). It may be noted that the 24 hr. values showed marked variations, but in some cases the radioactivities found for the alkali-soluble collagen fraction at 24 hr. exceeded significantly those found for any other protein in the same experiment.

The values obtained for the citrate-soluble and water-soluble non-collagenous fractions of the phosphate extracts of skin and those for plasma proteins were closely similar throughout. It follows that all these fractions must contain proteins of similar average turnover rates.

Table 5. *Radioactivities of DNP-glycine samples prepared from the skin and plasma proteins of female rabbits which had been fed* [α-^{14}C]*glycine by mouth*

Rabbit no.	Time between feeding of labelled glycine and killing (days)	Wt. of rabbits (g.)	Radioactivities of DNP-glycine (counts/min./sq.cm. at 'infinite thickness')					
			Plasma proteins	Water-sol. proteins of skin	Citrate-insol. proteins of skin	Alkali-sol. collagen	Acid-sol. collagen	Insoluble collagen
5	0·3	554	844	893	713	67·6	3·6	56·6
6	0·3	612	904	1051	1019	131·5	7·0	84·0
7	1·0	536	677	834	848	104·2	55·8	74·6
8	1·0	560	512	825	677	390	33·4	71·5
9	1·0	585	739	588	938	1362	150·8	83·3
10	1·0	490	782	542	562	682	41·6	37·3
3	3·0	605	—	—	157	61·0	86·3	41·8
4	3·0	640	475	380	351	73·5	172	74
1	7·0	530	135·1	—	108	20·8	106	68·4
2	7·0	550	151·5	—	97	19·8	142·9	128

— Sample lost or not isolated.

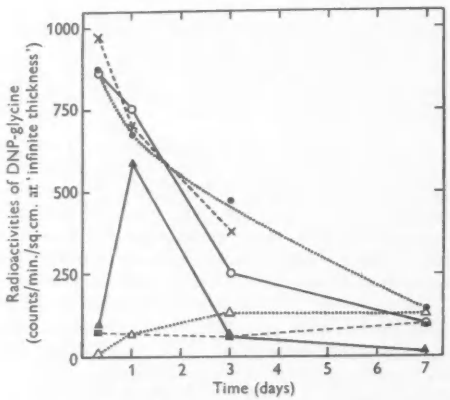

Fig. 3. Time/activity curves of proteins from the skin and plasma of rabbit. The averaged values of the radioactivities of the DNP-glycine samples given in Table 5 have been used to plot the above curves: ▲, alkali-soluble collagen; △, acid-soluble collagen; ×, water-soluble protein; ●, plasma proteins; ○, citrate-insoluble protein; ■, insoluble collagen.

DISCUSSION

The metabolism of proteins of the skin other than collagens

The present experiments show clearly that rabbit skin contains proteins of greatly differing renewal rates. A similar conclusion had already been reached by Ussing (1941), who investigated the incorporation into proteins of deuterium supplied as deuterium oxide. It is reasonable to assume that these varying turnover rates are somehow related to differences in the organized structures with which these proteins are associated. The skin preparations used consisted mainly of dermis, but contained also epidermis and may have been contaminated with small amounts of hypodermis. Both the dermis and epidermis contain a great variety of cells, but a large part of the total mass of the skin is not cellular. This 'extracellular' phase, which has been estimated to occupy in the rabbit about 55% of the total skin volume (Manery & Hastings, 1939), contains collagen as the quantitatively most important solid. In addition, it may be assumed that there will be present in this phase, as in other parts of the extracellular space, plasma proteins which have escaped through the capillary lining. The protein content of tissue fluids has never been directly measured, but good reasons have been put forward to suggest that the plasma protein concentration in the extracellular fluids of the limbs and the skin may be about 0·5–1%, i.e. similar to that of the peripheral lymph (Landis, 1934, 1946; Drinker, 1946). The protein fraction isolated in the present experiments after freeze-drying was found to resemble plasma proteins electrophoretically (Fig. 2). It represented about 0·4–0·5% of the total wet weight of the skin, or about 0·8% of the estimated extracellular mass. We may therefore assume that this fraction is indeed composed of plasma proteins and is derived mainly from the extracellular phase of the dermis. The fluid or gel between the collagen fibrils is generally considered to have a low protein content and this is supported by the fact that connective tissue such as tendon which has only a small number of cells contains very little non-collagen protein. It can be deduced, therefore, that those fractions of the phosphate extract of skin, which are not composed of alkali-soluble collagen or plasma proteins, i.e. the citrate-insoluble fraction and part of the water-soluble fraction, are derived mainly from the cells of the skin, probably largely from those of the dermis. The high radioactivities found for these fractions

shortly after feeding of the labelled glycine and the rates of decrease of radioactivity with time indicate that the cell proteins of the skin have turnover rates of the same order as those of the mixed proteins of the liver, kidney, etc. The very low overall renewal rates of skin proteins observed are thus caused mainly by the high collagen content of the skin. These low average figures will be particularly misleading, if glycine, which is present in such large amounts in collagen, is used as a label.

The metabolism of skin collagens

Acid-soluble and insoluble collagen. The results presented in Table 4, and similar observations reported by Orekhovich (1952), indicate that the first extract of acid-soluble collagen has a relatively high radioactivity soon after administration of labelled glycine; this activity decreases in successive fractions of this acid-soluble material and is lowest in insoluble collagen. Later, radioactivity becomes equal in the different fractions, until ultimately the order of activities is reversed and is highest in insoluble collagen. These findings have led Orekhovich (1952) to conclude that acid-soluble collagen is a precursor of insoluble or 'true' collagen and to suggest that the name 'procollagen' is indeed justified. It will be observed that, compared with the non-collagen proteins of skin, the radioactivities of acid-soluble collagen are very low, at least at periods up to 3 days after labelled glycine has been given. The activity/time curves of the various fractions of acid-soluble and insoluble collagen are of such a shape (Fig. 3) that no calculations of turnover values can be made, and it is doubtful whether the isotope data can be explained on the assumption that all insoluble collagen passes through the stage of acid-soluble collagen. Rabbits of the age used increase their body weight and probably also their total collagen content by approx. 2% per day. Since acid-soluble collagen was found to be about 20% of the total collagen, approx. 10% of the acid-soluble collagen would have to be synthesized per day and a similar amount would have to be converted into insoluble collagen. The activity/time curve to be expected on such a basis should thus resemble those of plasma or liver proteins; but this is obviously not the case.

It is felt that a more satisfactory explanation is the following: the two fractions, i.e. acid-soluble and acid-insoluble collagen are not distinct groups of proteins and both are obtained from fibres deposited outside cells. It is true that gelatin obtained from insoluble collagen has been found in the present experiments to have a higher tyrosine and lower hydroxyproline content than acid-soluble collagen and similar results have been reported by Bowes, Elliott & Moss (1953). But these workers have reported, on the basis of a comprehensive analysis, that there is a very close resemblance between these two protein fractions with respect to all other amino acids. Bowes *et al.* (1953) suggested that the insoluble collagen, identical in composition with acid-soluble collagen, is associated with some other substance, relatively rich in tyrosine and poor in hydroxyproline. It is also possible, and in our opinion more likely, that this tyrosine-rich material is not chemically linked with collagen in living tissue, but represents a contamination difficult to remove. The differences between these two collagen fractions would then not be of a chemical nature, but be due to physical factors affecting extractability by acidic solvents. The recent experiments of Jackson (1953) also indicate that there is no sharp division between acid-soluble and insoluble collagen. It is known (Gross, 1950) that in the young animal collagen fibrils are relatively thin and increase in size with age. The collagen of small, isolated fibres may be more readily extracted than the massive network found in the more adult animal. There may also be increasing aggregation of collagen molecules with age or increasing association with another substance (Bowes *et al.* 1953) affecting extractability with a citrate solution. The first extraction with weakly acidic solvents will thus remove mainly the collagen recently laid down, i.e. the material derived from small fibres, or from the outer layers of larger fibres. As extractions are repeated, more of the 'older' collagen will be made soluble, until an apparent limit is reached, due to denaturation or other unknown factors. Such an explanation fits satisfactorily the isotope results reported by Orekhovich (1952) and by ourselves. Indeed it is difficult to reconcile the simple precursor hypothesis with the finding that the maximum activity 'migrates' gradually with time from the first to the last extract of acid-soluble collagen and ultimately to insoluble collagen.

An association or contamination of insoluble collagen with a tyrosine-rich material would also explain the surprisingly high apparent radioactivity found for this collagen in some of the 8 and 24 hr. experiments of the second series (Table 5). This interpretation is supported by the observation that on fractionation of the gelatin, high tyrosine content and high radioactivity of the isolated glycine are apparently associated, at least with earlier collagen samples. It is clear that the gelatin from insoluble collagen is heterogeneous and is probably contaminated with a non-collagen protein of relatively high turnover rate.

Alkali-soluble collagen. This fraction was found to differ completely from the other two collagen fractions and indeed from all other proteins investigated. The radioactivity was high after 24 hr., but had decreased sharply at 3 days (Fig. 3). The

variations between the results obtained in altogether six 24 hr. experiments (Tables 4 and 5) were large, but this may be explained as follows: owing to differences between animals it is unlikely that the maximum activity in this fraction occurred in all experiments at the same time after injection. It is indeed doubtful whether in any of these experiments a value was obtained which was close to the maximum, particularly since both the ascending and descending parts of the activity/time curve appear to be very steep. On this basis, the high radioactivities at 24 hr. which exceeded, in three out of six experiments, those of any protein fraction measured, and the general character of the curve (Fig. 3) suggest that alkali-soluble collagen is formed and removed rapidly with a half-life of 2 days or less. Such a value is compatible with the assumption that this material is indeed a precursor of all other collagens of the skin. The initial solubility in disodium hydrogen phosphate solution suggests that the state of this alkali-soluble material in the tissue differs from that of the other collagen fractions. Its initial solubility may be due to a lower particle weight or to association with another substance. During isolation this association may be broken or polymerization may occur, leading to a change in solubility. We have no information about the morphological origin of this fraction. It may be suggested, tentatively, however, that alkali-soluble collagen is not derived from microscopically visible fibres, but may have been present either in the space between the fibres or even in or near the fibroblasts which are generally assumed to be responsible for the formation of collagen.

SUMMARY

1. The proteins present in the skin of growing rabbits have been separated into the following main fractions: (*a*) material soluble in phosphate buffer pH 9; (*b*) proteins insoluble in phosphate, but soluble in citrate pH 3·8; (*c*) collagen not extracted by these procedures, but converted into gelatin by autoclaving; (*d*) an insoluble residue.

2. Evidence was obtained that the phosphate-soluble material was a mixture of cellular proteins, plasma proteins derived from the extracellular space and a small amount of a collagenous protein ('alkali-soluble collagen'). The citrate-soluble fraction consisted of collagen-like material which is called acid-soluble collagen ('procollagen' of Orekhovich).

3. The amounts of hydroxyproline, glycine and tyrosine in the three collagenous fractions were similar, although there were some significant differences. Evidence was obtained that gelatin from insoluble collagen is either heterogeneous with respect to amino-acid composition or contaminated with protein of non-collagen type.

4. Experiments using a single dose of [α-^{14}C]-glycine and isolating the glycine as the 2:4-dinitrophenyl derivative showed that the proteins of the phosphate-soluble fraction, other than alkali-soluble collagen, had turnover rates similar to those of plasma and liver proteins. The highest radioactivity of the alkali-soluble collagen was observed about 24 hr. after administration of the labelled glycine and exceeded in some experiments the maximum values found for other proteins of the phosphate fraction or plasma proteins. Thereafter it dropped rapidly to values below those of acid-soluble or insoluble collagen.

5. The radioactivity of the glycine of the acid-soluble collagen by comparison with that of alkali-soluble collagen increased and decreased very slowly and the activity/time curve of insoluble collagen was almost flat.

6. It is concluded that alkali-soluble collagen is almost certainly a true precursor of the other collagen fractions. The metabolic role of acid-soluble collagen is more uncertain and its activity/time curve suggests that it is not a necessary intermediate in the formation of all the insoluble collagen of the skin.

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Content of Vitamin A_2 in some Nile Fishes

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Heilbron, Gillam & Morton (1931) observed that in the antimony trichloride colour test, an absorption band at 693 mμ. accompanied the 617 mμ. band in many fish-liver oils. For several years, little progress was made in characterizing the new chromogen. It accompanied vitamin A in the richest concentrates and no separation could be effected either by chromatography or molecular distillation. Edisbury, Morton & Simpkins (1937) found relatively large quantities of the 693 mμ. chromogen in the eyes of gold-fish and the livers of trout. They therefore suggested that the 693 mμ. chromogen should be designated vitamin A_2, because its distribution suggested that it could replace vitamin A, particularly in fresh-water fish.

Morton, Salah & Stubbs (1946) prepared vitamin A_2 free from vitamin A_1, and later Morton, Salah & Stubbs (1947) suggested that it was dehydrovitamin A_1, with the extra double bond in the β-ionone ring.

From the retinas of marine fishes, Wald (1935) obtained rhodopsin from which, after bleaching, retinene$_1$ could be extracted by light petroleum. An analogous pigment, porphyropsin, was obtained from the eyes of fresh-water fishes (Wald, 1936); from this retinene$_2$ could be obtained. Morton & Goodwin (1944) proved that retinene$_1$ is vitamin-A_1 aldehyde and retinene$_2$ was later shown to be vitamin-A_2 aldehyde (Morton *et al.* 1947).

Wald (1937) has made the interesting observation that, in general, the eye tissues of fresh-water fishes contain vitamin A_2, while those of marine fishes contain vitamin A_1. Fishes which migrate from the one environment to the other contain both vitamins. It is with these facts in mind that Nile fishes have been examined. The amounts of both vitamin A_1 and A_2 in such fishes have not hitherto been determined. The results may help in accounting for distribution of vitamins A_1 and A_2 in nature.

EXPERIMENTAL

Materials. The fishes reached the laboratory within a few hours of being caught and were immediately dissected. The livers were removed and wet weights recorded.

Extraction of oils. The oils were prepared by grinding the livers with silver sand and anhydrous Na_2SO_4 and extracting with light petroleum 3 or 4 times. The solvent was completely distilled off in an atmosphere of CO_2 and the residue was weighed. The light petroleum used was purified by refluxing for 30 min. with $KMnO_4$ and dilute H_2SO_4; washed with water, then NaOH and water, and then dried over anhydrous $CaCl_2$. It was then fractionated and the fraction boiling between 40 and 60° was used.

Alumina. Alumina for chromatography (British Drug Houses Ltd.) was weakened by the addition of 10% of its weight of water and used immediately. The following is the procedure which was found suitable for weakening the active alumina. Alumina (10 g.) is made into a paste with

light petroleum and 1 ml. of water is added dropwise while the paste is triturated in a mortar. The alumina is kept under light petroleum and used the same day. In this way, the alumina produced was found suitable for chromatography and had no detrimental effect on the vitamin A present.

Maleic anhydride. This was recrystallized from benzene and then dissolved in A.R. benzene to give a 10% (w/v) solution.

Antimony trichloride reagent. Anhydrous antimony trichloride (20%, w/v) was dissolved in chloroform previously dried over $CaCl_2$.

Spectrophotometry. A Unicam Photoelectric Spectrophotometer was used for the ultraviolet examinations, and a Hilger-Nutting Visual Spectrophotometer to locate the position of the bands in the antimony trichloride colour test.

Method of analysis of vitamin A in oil. Two methods were applied.

(*a*) A solution of the oil in $CHCl_3$ was prepared at a known concentration, such that when 0·2–0·4 ml. was treated with 4 ml. of antimony trichloride reagent the absorption of the blue solution at 617 and 693 mμ. could be measured with the Hilger-Nutting Visual Spectrophotometer. Vitamin concentrations were calculated on the basis that vitamin A_1 has an $E^{1\%}_{1\,cm.}$ 617 mμ. of 5000 and vitamin A_2 has an $E^{1\%}_{1\,cm.}$ 693 mμ. of 4000.

(*b*) After chromatography and suitable dilution, the absorption curve was measured in the Unicam Spectrophotometer. After correction for irrelevant absorption (Morton & Stubbs, 1946) had been made, the potency was calculated on the basis of $E^{1\%}_{1\,cm.}$ 325 mμ. = 1700 for vitamin A_1 and $E^{1\%}_{1\,cm.}$ 348 mμ. = 1400 for vitamin A_2. The following values were used in correcting curves corresponding with a predominance of vitamin A_2:

$$E_{max.} = 348\text{–}350\ m\mu.,\ E_{330\ m\mu.} = E_{366\ m\mu.}$$

and

$$E_{348\ m\mu.}/E_{330\ m\mu.} = 1{\cdot}18.$$

These figures were obtained from a vitamin-A_2 curve on material seven-times chromatographed on 10% watered alumina.

Method of determining neovitamin A. The method is essentially that of Meunier & Jouanneteau (1948) which depends on the much more rapid formation of an adduct of maleic anhydride with *all-trans*-vitamin A than with *neo*-vitamin A.

A weighed portion of the oil was dissolved in benzene. Suitable dilution gave a solution in benzene containing 200–300 i.u./ml. 5 ml. of this solution were pipetted into a flask and 5 ml. of maleic anhydride reagent added. After being shaken, the mixture was kept in the dark at 15° for 16 hr. Exactly 1 ml. was diluted for use in the antimony trichloride colour test. A measured volume of the untreated solution was tested in the same way. The colour was much weakened in the solution treated with maleic anhydride. The percentage of vitamin A existing in the *neo* form is given by the formula $\frac{R - R_1}{R_2 - R_1}$ where R = % recovery of vitamin A in the sample; R_1 = that of vitamin A using a pure *all-trans*-vitamin A ester; R_2 = that of *neo*vitamin-A ester in a sample of ester free from *all-trans*-vitamin-A ester, the criterion of recovery being the appearance of the 620 mμ. maximum in the colour test.

The values of R_1 and R_2 given by Meunier & Jouanneteau are 2 and 90, respectively. These same figures were also used for vitamin A_2; in the absence of direct calibration of *neo*vitamin A_2, it has been assumed that *neo*vitamin A_1 and *neo*vitamin A_2 behave almost identically.

RESULTS AND DISCUSSION

The results of the determination of vitamin A_1 and A_2 and *neo*vitamin A_2 in a number of Nile fish-liver oils are given in Table 1. This shows the vitamin-A content in the livers of sixteen species of Nile fishes. The survey has been limited to these species because they are the common varieties in Egypt and those which are consumed in preference to others by Egyptians.

The results show that Nile fishes, with the exception of *Anguilla aegyptica* and *Tilapia nilotica*, contain variable amounts of vitamin A_2 per g. liver oil, which amounts are, however, characteristic of the species. It was noticed that several species had a high vitamin-A_2 content, although the amount

Table 1. *Vitamin-A content of liver oils of Nile fishes*

Species*	Egyptian name	Weight in g. of fish	Oil in liver (%)	Vitamin A_1 (μg./g. oil)	Vitamin A_2 (μg./g. oil)	*Neo*vitamin A_2 (% approx.)
Tilapia nilotica L.	Bolti	75–450	4–13	320–600	—	—
Anguilla aegyptica Shaw	Hannash	90–520	2–3	4000	—	—
Barbus bynni Forsk.	Bynni	220–1260	2·5–8	—	1430	17·3
Labeo coubie Rüpp.	—	220–660	2–6	—	840	—
Labeo niloticus Forsk.	Lebees	122–425	1–5	—	2600	—
Mormyrus kannume Forsk.	—	72–520	1·4–4·2	—	10 000	17·5
Lates niloticus L.	Homar	145–250	1–4	—	3000	20·2
Chrysichthys auratus Geoff.	Zammar	45–50	2·2–4·5	—	1230	21·5
Clarias lazera C. & V.	Karmout	95–440	1·5–9·4	—	3250	—
Malopterurus electricus Gn.	Raad	500–2000	1·6–3·4	—	13 000	—
Synodontis schall Bl. Schn.	Annuma	122–240	1·3–4·9	—	2500	—
Schilbe mystus L.	Shelba	65–142	2–5	—	70 000	—
Bagrus bayad Forsk.	Bayad	72–640	0·3–1·6	—	30 000	16·2
Hydrocyon forskalli Cuv.	Kelb-el-Bahr	440–1870	1·5–6	—	40 000	—
Alestes dentex L.	Rhy	55–525	2–15	—	4000	22·5

* See Boulanger (1907).

increased with the size of the fish. The presence of vitamin A_1 in *Anguilla aegyptica* and *Tilapia nilotica* is explained on the basis that the first is a migratory fish and the second lives mainly in the Delta region. It is interesting to notice that *Tilapia* is the fish most frequently appearing in the graphic and plastic representations of the ancient Egyptians.

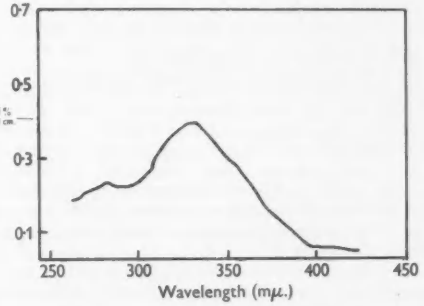

Fig. 1. Absorption spectrum of the liver extract in light petroleum from *Tilapia nilotica*. Maxima at 330 and 285, $E^{1\%}_{1\,cm.}$ 0·36 and 0·22.

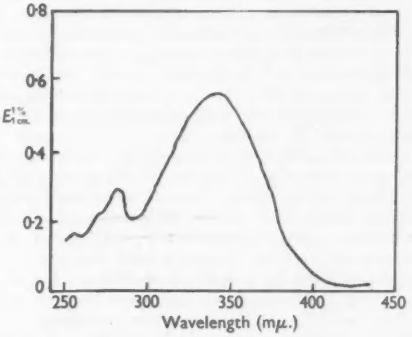

Fig. 2. Absorption spectrum of liver extract in light petroleum from *Hydrocyanon forskali*. Maxima at 345 and 285, $E^{1\%}_{1\,cm.}$ 0·53 and 0·26.

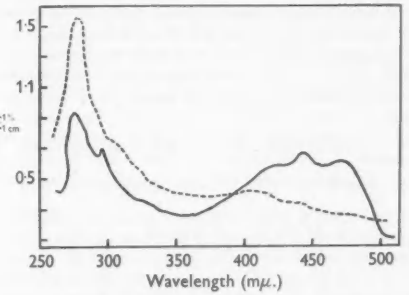

Fig. 3. Light petroleum extracts of stomach and intestine of *Clarias lazera*. - - - -, stomach; ——, intestine.

Several samples of the various species were examined in the Beckman Spectrophotometer. Only *T. nilotica* and *Anguilla aegyptica* oils had a maximum at 330 mμ., indicating the presence of vitamin A_1. But the 285 mμ. band which is characteristic of vitamin A_2 was invariably present as shown in Fig. 1. The other species gave oils with a maximum at 245 mμ., together with another peak at 285 mμ. There was not a single exception. The oils of *Hydrocyanon forskali* and *Bagrus bayad* are exceptionally rich. Fig. 2 shows the absorption curve of *Hydrocyanon forskali*.

It was found that almost all the vitamin A_1 and A_2 occurred in the liver in the ester form. The vitamin-A_2 alcohol obtained after saponification gave an absorption spectrum in striking agreement with that of vitamin A_2 alcohol prepared by Ponndorf reduction of retinene$_2$.

The stomach and intestinal contents of *Clarias lazera* and *Lates niloticus* were examined spectroscopically and found to contain a mixture of carotenoids, maxima being recorded at 420, 440 and 470 mμ., as shown in Fig. 3 for *Clarias lazera*.

SUMMARY

1. The vitamin-A_1 and -A_2 contents of the livers of a number of species of Nile fishes have been determined.

2. The presence of *neo*vitamin A_2 has been demonstrated.

3. Appreciable amounts of carotenoids did not occur in the livers of fishes, but carotenoids were present in the stomach contents of two species studied.

We are greatly indebted to Dr Abu Samra of the Royal Biological Institute for Marine Research for the supply of samples, and to Prof. R. A. Morton for encouragement and help.

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Studies in Immunochemistry

13. PREPARATION AND PROPERTIES OF THE 'O' SOMATIC ANTIGEN OF *SHIGELLA DYSENTERIAE* (SHIGA)

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The isolation and properties of an antigenic material which possesses the immunological character of the 'O' antigen of *Shigella dysenteriae* (Shiga) was described in a series of papers (Morgan, 1937, 1939, 1949; Morgan & Partridge, 1940, 1941; Partridge & Morgan, 1940). The results of this work indicated that the 'O' antigen consisted of a labile, polymolecular complex which contained three main components; polysaccharide, conjugated protein and phospholipid. It was pointed out that although the results of the examination of the preparation indicated that the isolated antigenic material was largely freed from unspecific contaminants, the lack of knowledge of the behaviour of large molecular aggregates when subjected to the relatively crude and inefficient methods of purification employed did not enable a final decision as to the degree of homogeneity of the material to be reached.

The object of the present communication is to present the results of a re-examination of the problem of the isolation and characterization of the 'O' antigen of *Sh. dysenteriae* which includes a detailed study of the materials obtained by high-speed differential centrifugation.

MATERIALS AND METHODS

Organisms. Sh. dysenteriae (Shiga) (Type 1 of Kauffmann, 1951), strain K. 624 'Smooth' was used for the extraction of antigen, this strain had previously been used for this purpose by Morgan (1937) and Morgan & Partridge (1940, 1941). Cultures were carefully checked for purity before each large-scale growth, for suspension stability, and for smoothness of colonial morphology by plating on tryptic meat-digest agar. 'Rough' colonies were very rarely encountered in this strain.

Materials for analysis. For analysis, samples of extraction products were thoroughly dialysed against distilled water at 0–2° and dried from the frozen state. They were further dried to constant weight *in vacuo* at 78° before making into solutions of known (%, w/v) concentration in water. The freeze-dried materials absorbed up to 15% of their weight of moisture when left exposed to the air.

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Total nitrogen. This was determined by the Kjeldahl method using the distillation apparatus of Markham (1942) and the mixed bromocresol green + methyl red indicator of Ma & Zuazaga (1942).

Phosphorus estimation. This was determined on samples containing 5–20 μg. of P by a modification of the Fiske & Subbarow (1925) method.

Sugars. Glucosamine was estimated by a modification of the method of Elson & Morgan (1933) on samples after hydrolysis with 0·5 N acid. Rhamnose was determined by the method of Dische & Shettles (1948) on samples without previous hydrolysis.

Ultracentrifugation. Preparative ultracentrifuge runs were carried out in a Spinco Model 'L' Ultracentrifuge at 2–4°. Figures quoted for the force in *g* refer to the forces exerted at the centres of the tubes and represent average values. Analytical ultracentrifuge runs were carried out in a Spinco Model 'E' machine.

Antigenicity. Measured by the capacity of the materials to elicit the formation of specific *Sh. dysenteriae* (Shiga) agglutinins in the rabbit. Animals were injected intravenously with 0·5 ml. samples of fractions dissolved in sterile saline, generally three doses were given at 4-day intervals and the sera obtained a week after the last injection tested for the presence of agglutinins using a suspension of the heat-killed homologous organisms. The degree of agglutination was read after incubating the test serum and suspension for 2 hr. at 55° and leaving overnight at 0–2°. Normal rabbit sera rarely show agglutinins above a dilution of 1 in 2. This test was used to determine in which fraction the antigenic material was present through the purification process.

Toxicity. Toxicity was estimated by injecting groups of ten white mice (body wt. 18–22 g.) intraperitoneally for each of six dilutions of the test material in sterile saline. Deaths were recorded over 4 days, most of the animals which died did so on the second day.

EXPERIMENTAL AND RESULTS

Isolation of the somatic antigenic complex

Preparation of bacteria for extraction. The organisms were grown on solid 'CCY'-agar (Gladstone & Fildes, 1940) in enamelled steel trays, each containing 500 ml. of medium. After 22 hr. growth at 37°, the bacteria were reaped, filtered and washed quickly with saline and with water at 0–2°. The centrifuged cells were resuspended and killed by pouring into 10 vol. of cold acetone. After being checked for

sterility, the acetone was changed several times, the organisms were collected by centrifugation and dried *in vacuo*. Yield, about 400 mg. of dried organisms from each tray.

Extraction with diethylene glycol. The method used for the extraction of the antigenic material was essentially the same as that described by Morgan (1937) and by Morgan & Partridge (1940). In order to determine if the treatment of the organism with acetone before extraction with diethylene glycol, as used in the earlier work, had any deleterious influence on the yield or quality of the 'O' antigen, one preparation of living *Sh. dysenteriae* was dried from the frozen state and extracted directly with diethylene glycol. The yield and properties of the material soluble in the organic solvent did not appear to differ in any way from the product which was obtained when acetone-killed culture was employed. The technical difficulties and dangers associated with the production on a large scale of freeze-dried, living, virulent bacteria have not been satisfactorily overcome, and this type of culture preparation has, therefore, not been extensively used.

Acetone-dried bacterial cells (170 g.) were extracted with 10 parts (v/w) of freshly redistilled diethylene glycol. The suspension was shaken mechanically for several hours each day for 3–4 days at room temperature, methanol (10%, v/v) was added to reduce the viscosity and the cells were removed in the closed bowl of a Sharples centrifuge. The clear, yellow supernatant solution was passed through a Berkefeld filter candle, dialysed against distilled water at 2–4° until free from glycol, and the opalescent aqueous solution obtained was concentrated under reduced pressure at 16° until the solid content was about 1%.

The sedimented bacteria recovered from the Sharples bowl were resuspended in fresh diethylene glycol and re-extracted; four extracts were made. After the first extraction the cells were much more easily sedimented, and less glycol was used in the third and fourth extractions.

In order that the products of successive extractions could be compared, each was handled separately through the purification process.

Fractionation with acetone. Each of the four extracts was fractionated with acetone in the same way. The aqueous solution was cooled to 0–2° and acetone at −10° added slowly to a final concentration of 50% by vol. Some material was precipitated at this level and was removed by centrifugation. The acetone concentration was then increased to 66%, at which level the bulk of the antigenic material was precipitated. The precipitate was redissolved in water and treated with acetone a second time, a small amount of material was again removed at the 50% level and the antigen precipitated by increasing the concentration to 60%. The solution in water and precipitation with acetone was repeated a third time and the 50–60% acetone fraction finally obtained was redissolved in water, dialysed in the cold against distilled water and dried from the frozen state.

The yields, and the total N and total organic P contents of the four extracts are shown in Table 1. No inorganic P was present.

The material at this stage showed a definite absorption in the ultraviolet region at 260 mμ., which was due to the presence of nucleic acid and nucleoprotein. These materials could be removed by fractionation with $(NH_4)_2SO_4$.

Fractionation with ammonium sulphate. The products of the acetone fractionation of each of the four diethylene glycol extracts were dissolved separately in distilled water to give 1% solutions, and each was fractionated with $(NH_4)_2SO_4$. Saturated $(NH_4)_2SO_4$ soln. at 20° was added to the antigen solutions at the same temperature and the precipitates formed were collected by centrifugation, washed with $(NH_4)_2SO_4$ soln. of the same percentage saturation as that which had precipitated the fraction and the washings were added to the appropriate supernatant solution. The fractionation scheme is set out in Fig. 1, from which it will be seen that each extract was divided into five parts separating

Table 1. *The 50–60% (v/v) acetone fractions of 'Shiga' diethylene glycol extracts*

No. of diethylene glycol extract	Yield (g.)	Total N (%)	Total P (%)	N/P
1	3·02	4·4	1·4	3·1
2	2·80	4·2	1·4	3·0
3	1·87	4·2	1·5	2·8
4	1·11	5·1	1·6	3·2

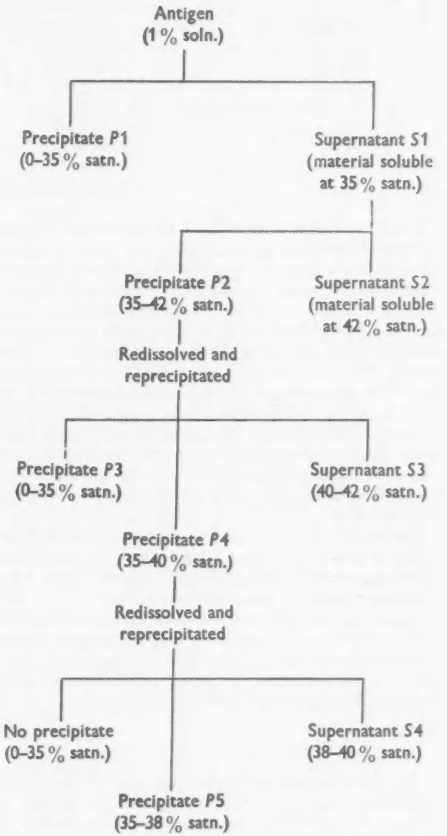

Fig. 1. Ammonium sulphate fractionation of the 50–60% (v/v) acetone fractions of 'Shiga' diethylene glycol extracts.

Table 2. *The ammonium sulphate fractionation of the* 50–60 % (*v/v*) *acetone fractions of diethylene glycol extracts of* Shigella dysenteriae (*Shiga*)

		Diethylene glycol extract			
		1st	2nd	3rd	4th
Original material	Wt. (g.)	3·0	2·8	1·8	1·0
	N (%)	4·4	4·2	4·2	5·1
	P (%)	1·4	1·4	1·5	1·6
Fractions					
*P*1 + *P*3 (0–35 %)*	Wt. (g.)	0·40	0·26	0·22	0·05
	N (%)	3·9	3·6	3·1	5·3
	P (%)	1·1	0·9	1·2	0·9
	N/P	3·5	4·0	2·7	5·6
*P*5 (35–38 %)*	Wt. (g.)	1·50	0·85	0·76	0·40
	N (%)	4·4	4·6	4·3	4·5
	P (%)	1·0	1·1	1·2	1·0
	N/P	4·4	4·2	3·6	4·5
*S*4 (38–40 %)*	Wt. (g.)	0·37	1·30	0·09	0·04
	N (%)	3·9	4·6	3·8	3·3
	P (%)	1·1	1·2	1·1	0·9
	N/P	3·5	4·0	3·5	3·6
*S*3 (40–42 %)*	Wt. (g.)	0·33	0·09	0·36	0·36
	N (%)	3·4	3·6	3·6	3·4
	P (%)	1·2	1·1	1·2	1·4
	N/P	2·8	3·3	2·9	2·4
*S*2 (soluble at 42 %)*	Wt. (g.)	0·11	0·09	0·08	0·03
	N (%)	5·9	6·2	6·6	6·3
	P (%)	1·4	2·2	2·6	2·5
	N/P	4·1	2·3	2·5	2·5

* % Saturation with ammonium sulphate.

at saturation levels of: below 35, 35–38, 38–40, 40–42 %, and that soluble at 42 %. Each fraction was dialysed at 0–2° until free from salt, and recovered by drying from the frozen state. The weights of the fractions obtained, together with their N and P contents, are given in Table 2. The materials precipitated at 35 % and those remaining in solution at 42 % saturation of salt were unstable in aqueous solution after recovery and both materials showed a strong ultraviolet absorption at 260 mμ., these fractions *P*1, *P*3 and *S*2 accounted for a third of the weight of the product of acetone fractionation. The other fractions gave stable opalescent aqueous solutions from which no material sedimented on prolonged centrifugation at 3000 rev./min. They showed no ultraviolet absorption which could be attributed to nucleic acid.

The four *S*4 fractions and the four *P*5 fractions had very similar N and P contents and were, therefore, pooled and reprecipitated with $(NH_4)_2SO_4$; no material came out of solution at the 35 %-saturation level and only a little remained in the 40 %-saturated supernatant fluid. The 35–40 % fraction was then precipitated twice with acetone, each time the 50–60 % (v/v) acetone fraction was kept and after dialysis and freeze-drying 4 g. was recovered, which contained 4·35 % N and 1·16 % P.

Extraction on a larger scale

Dry bacterial cells weighing 1 kg. were extracted four times with diethylene glycol by the method already described. In order that larger apparatus and vessels than one normally encounters in the laboratory would not be required, the batch was split into four parts, *A*, *B*, *C* and *D*, each weighing 250 g.

To avoid the difficulty of dialysing large volumes of diethylene glycol extracts and the subsequent concentration of the resulting dilute aqueous solutions, the extracts cooled to 0–2° were poured into several volumes of acetone at −15°, the acetone being recovered for use again. The precipitated material was redissolved in water to yield a 1 % (w/v) solution and fractionated with acetone as already described above. A yield of 99 g. of crude antigen was recovered between the concentrations of 50 and 60 % (v/v) acetone.

The acetone products were made up into batches of suitable size and fractionated with $(NH_4)_2SO_4$ as described above, except that the materials were collected in only three fractions, that precipitated at 35 % saturation, between 35 and 40 %, and that soluble in 40 %-saturated $(NH_4)_2SO_4$ solution. These materials were recovered by freeze-drying after dialysis in the cold against de-ionized water ('Permutit' de-ionizer). The main fraction precipitated in the range of 35–40 % saturation with $(NH_4)_2SO_4$ was again precipitated with acetone in the range of 50–60 % (v/v) and the product finally obtained weighed 53 g.

Examination of the materials obtained by fractionation with ammonium sulphate. Samples of the three fractions, dissolved in dilute alkali, were examined for absorption in the ultraviolet region. Both the side fractions showed strong absorption at 260 mμ. due to nucleoprotein (fraction below 35 %) and nucleic acid (fraction above 40 %), but the main fraction showed no maximum in this region. It had, however, a definite absorption at 245 mμ. and a weak one at 295 mμ. The absorption curves are shown in Fig. 2.

An examination of this material by a fractional-solubility test, as described later, showed that it was not homogeneous, and photographs obtained in the analytical ultracentrifuge

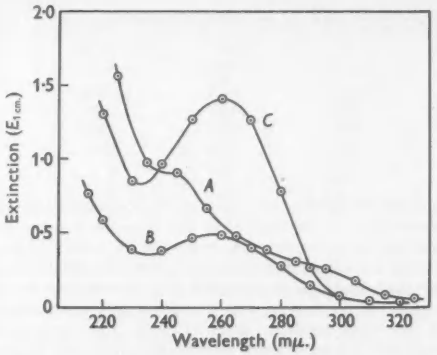

Fig. 2. The ultraviolet absorption spectra of the products of ammonium sulphate fractionation of 'Shiga' diethylene glycol extract (50–60%, v/v, acetone fraction). *A*, 35–40% fraction; *B*, material precipitated below 35%; and *C*, that remaining in solution at 40% saturation. *A* at 0·05%, *B* and *C* at 0·01% concentration. All materials in 0·01 N-NaOH.

showed that two components were present, one a rapidly sedimenting, polydisperse component and the other a slowly moving, sharp peak. Before attempting to separate these materials, however, the phospholipid component was removed.

Removal of the phospholipid

Morgan & Partridge (1940) showed that the antigenic complex contained a phospholipid component which could be readily dissociated and removed by the action of formamide without destroying the capacity of the material to induce the formation of specific agglutinins. A method used originally by Miles & Pirie (1939) for removing the material 'PLI' (a lipid + phospholipid mixture) from the *Brucella* antigen was, in the present experiments, employed in place of formamide to remove the phospholipid.

Material which had been fractionated with acetone and $(NH_4)_2SO_4$, and shown to be free from detectable amounts of nucleic acid, was dissolved in water to 1% concn., cooled to 0–2°, and poured into 10 times its volume of ether:ethanol (1:1, v/v) mixture containing 0·5% of conc. HCl, the solvent mixture being kept at −10°. The 'Shiga' antigen was precipitated and recovered by centrifugation at 0–2°, this was redissolved in water at 0°, the precipitation process repeated twice and the phospholipid-free material recovered. From 48 g. of antigen treated in this way, 41·5 g. was obtained which contained 4·35% N and 0·6% P.

Fractionation by differential ultracentrifugation

The phospholipid-free antigenic material (32 g.) was dissolved in 2·5 l. of water and fractionated by differential centrifugation. The solution was first centrifuged for 2 hr. at 40000 *g* and the sediment (*P*1) was redissolved and freeze-dried. The supernatant fluid (*S*1) was centrifuged again for 2 hr. at 80000 *g* the sediment (*P*2) was similarly recovered and the remaining solution (*S*2) centrifuged for a further 3 hr. at 105000 *g*, giving a sediment *P*3. The supernatant solution remaining in the centrifuge tubes showed a sharp boundary which separated a lower opalescent part, removed as fraction (*S*3), from an upper part (*S*3*a*) which was clear. The results of the analysis of these five fractions for N and P suggested a further series of centrifugations in which fraction *P*2 was separated into a sediment *P*2*P* and supernatant *P*2*S* in 2 hr. centrifugation at 44000 *g*, *S*3 was separated into *S*4 and *P*4 at 105000 *g*, and *S*3*a* into sediment *P*5, and fractions *S*5*a* to *S*5*c*, being the top, centre and lower parts, respectively, of the supernatant solution, after a further 3 hr. at 105000 *g*. This fractionation scheme is set out in Fig. 3 with the weights and the N and P contents of the recovered fractions. Fractions *P*3 and *P*2*S* were pooled to give a product (*SpM*) having N, 4·46; P, 0·75% and weighing 9·8 g. A further 4·4 g. of antigenic

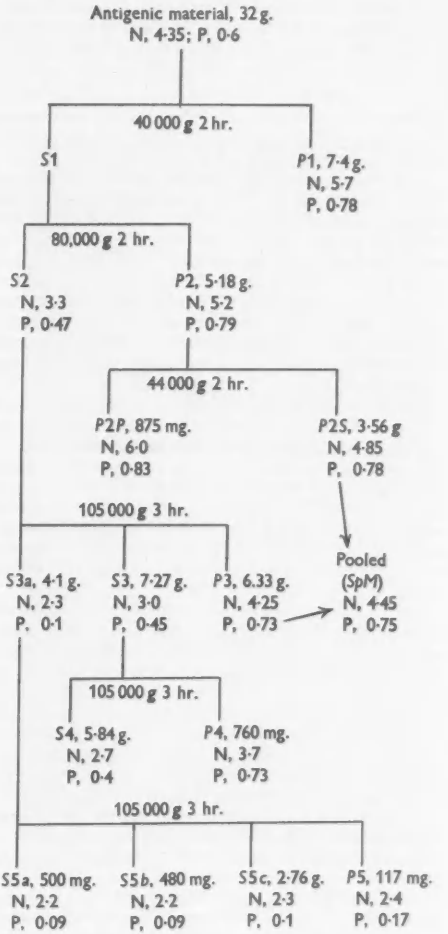

Fig. 3. Fractional centrifugation of 'Shiga' antigen. N and P figures are given as wt. %. For explanation see the text.

Table 3. *Materials derived from antigen preparation* SFa *by ultracentrifugation*

Fraction	Wt. (g.)	Recovery (%)	N (%)	P (%)	N/P
Original material	4·40	—	4·1	0·63	6·5
Below 25000 *g*	0·36	8	6·35	0·90	7·0
25000 to 105000 *g*	2·47	56	4·6	0·88	5·2
Over 105000 *g*	1·53	35	2·3	0·19	12·1

Table 4. *Fractionation of 'heavy' material (fractions* P1 *and* P2P*) by differential centrifugation*

Fraction	Wt. (g.)	Wt. (%)	N (%)	P (%)	N/P
*P*1/1	0·59	8	6·9	0·88	7·85
*P*1/2	0·39	5	6·4	0·85	7·5
*P*1/3	2·27	31	6·2	0·85	7·3
*P*1/4	4·14	56	5·2	0·75	6·7

material (*SFa*), after removal of phospholipid, was fractionated in the centrifuge in a simpler way and gave the products detailed in Table 3.

It is evident that, after fractionation with acetone and $(NH_4)_2SO_4$ and removal of the ether-soluble phospholipid, the antigenic material contains three components. The first of these is not sedimented at 100000 *g* under the conditions studied and contains about 2·2% N. This fraction has been purified and obtained in an homogeneous condition as shown by the methods of fractional solubility, electrophoresis and ultracentrifugation, and will be described in greater detail in a later communication; it is the specific, undegraded polysaccharide, free from the other components which make up the native antigenic complex. The material is not antigenic in the rabbit and not significantly toxic, and differs in some important respects from the undegraded polysaccharide which can be obtained from the antigenic complex by dissociation (Morgan & Partridge, 1941). The second material contains 4·5–4·6% N, sediments between 40000 and 105000 *g*, gives a stable, opalescent aqueous solution, and is a powerful antigen. The third material contains about 6% N, is thrown out of solution at 40000 *g* and fails to give a stable solution when resuspended in water.

Two of the most readily sedimented 'heavy' fractions (*P*1 and *P*2*P*, Fig. 3) were pooled, giving 7·45 g. of material containing N, 5·7; P, 0·79%. This was fractionated by differential centrifugation and gave four fractions, *P*1/1 to *P*1/4. *P*1/1 sedimented at 5000 *g*, *P*1/2 at 10000 *g*, while *P*1/3 and *P*1/4 were the heavily opalescent bottom half, and the almost-clear top half of the supernatant solution, respectively. The weights and the N and P contents of these fractions are shown in Table 4. Fraction *P*1/2 (300 mg.) was suspended in water and, after being allowed long enough for as complete solution as possible, was centrifuged at 5000 *g*. The opalescent supernatant solution was removed, the sediment resuspended in water and centrifuged again. This process was repeated three times in all. The final supernatant was clear and did not appear to have any material contained in it. However, the pooled supernatant solutions subsequently yielded 72 mg. of material which contained N, 4·25; P, 0·9%, and which was closely similar to the soluble antigen. The final water-insoluble fraction weighed 193 mg. and contained N, 7·1; P, 0·8%. A sample of this material (93 mg.) was suspended in 1% (v/v) acetic acid and heated for 3 hr. at 100°, after this time the material remaining in suspension was removed by centrifugation, leaving a clear solution from which the degraded specific polysaccharide was precipitated with 10 times its vol. of ethanol; 21 mg. was recovered, or about 23% of the weight of the water-insoluble material used. The amount of degraded polysaccharide recoverable from the soluble antigen (N, 4·5%) after hydrolysis under similar conditions is 65–70% (see below).

Examination of the main antigenic fraction (SpM)

A sample (400 mg.) of fraction *SpM* (Fig. 3) was examined for homogeneity in a fractional solubility test as follows. The material was suspended in 100 ml. of redistilled propylene glycol, in which it is sparingly soluble, and the suspension shaken mechanically. After 24 hr., when part had dissolved, the suspension was centrifuged and the supernatant solution removed. A fresh volume of solvent was added to the sediment and the process repeated until nearly all the material had dissolved. After 12 days, the material had been divided into 12 fractions, which were pooled in successive pairs and the materials recovered by pouring each dissolved fraction into several times its volume of acetone, washing until free from glycol and drying from the frozen state. Analytical figures for these fractions are shown in Table 5.

No significant differences can be seen between the analyses of the different fractions, whereas before fractional centrifugation, although no differences were found in the different fractions obtained in a solubility test using water as solvent, a significant rise was found in the N content of successive fractions in propylene glycol. This test, therefore, does not reveal any evidence of lack of homogeneity in the material investigated.

The material, *SpM*, was examined electrophoretically at pH's 4·1 and 8·0. The substance travelled very slowly and showed a small amount of a second component. Examination of the material in the analytical ultracentrifuge also showed that two components were present both at pH 4 and at pH 8. The major component travelled fast and was somewhat polydisperse. The second material, present in small amount only, gave a slowly moving, sharp peak, later identified as free, undegraded polysaccharide.

Table 5. *A fractional solubility test on antigenic material (fraction* SpM*) using propylene glycol*

Fraction	Wt. (mg.)	N (%)	P (%)	N/P	Rhamnose (%)	Glucosamine (base) (%)
1	109	4·4	0·75	5·9	22·4	14·6
2	215	4·4	0·75	5·9	22·2	14·6
3	125	4·3	0·74	5·9	22·9	14·6
4	120	4·55	0·75	6·05	21·8	13·6
5	115	4·4	0·75	5·9	21·7	14·2
6	248	4·55	0·78	5·85	21·7	14·0

Table 6. *Materials obtained by fractional centrifugation of pooled antigen preparations*

Fraction	Sedimentation range (*g*)	Wt. (g.)	N (%)	P (%)
Sp 2/3	25000–80000 (sedimented in 6 hr.)	1·25	4·53	0·83
Sp 3/1	80000–105000 (sedimented in 3 hr.)	1·05	4·56	0·82
Sp 3/2/1	105000 (supernatant solution from *Sp* 3/1 sedimented in 6 hr.)	1·50	4·50	0·80

Further fractional centrifugation

Fractions *SpM* (Fig. 3), *SFa* (middle fraction, Table 3) and *P*1/4 (Table 4), totalling 12 g., were pooled, dissolved in water, and centrifuged in such a way as to give three main antigen fractions sedimenting in different ranges of *g*, and such that each of the fractions was centrifuged at least twice at the lower force to remove the heavier material and at least 3 times at the higher force to remove lighter materials. Analytical details of these three fractions are given in Table 6. The amounts of material obtained in these fractions do not represent the amount present in the starting material, the remainder, present in the side fractions, was not recovered in this series of runs owing to the amount of centrifuging this would have entailed. The analysis of these fractions shows that although the antigenic complex is polydisperse, materials sedimenting at different levels of *g* and differing, therefore, in particle size, have most probably the same chemical composition.

Examination of materials for homogeneity

Fraction *Sp* 3/1 (Table 6) was examined for homogeneity in the analytical ultracentrifuge (Fig. 4). At 0·5% concentration and pH 4·5, the material showed only one component, but at 1·25% concentration a slower-moving, second component was still visible (Fig. 4*b*), though present in very small amount. At pH 8·0 the amount of this material, which was identified as free polysaccharide, had increased to several per cent (Fig. 4*c*). At pH 11 the free polysaccharide had increased still further in amount (Fig. 4*d*) and the separation of a further material from the main component is revealed. The amount of free polysaccharide present, at least in the physiological range of pH, is so low that it cannot be detected by other means. Further centrifugation of a sample of fraction *Sp* 3/1 gave a material (*Sp* 3/1/1) which showed no detectable change in N and P content but in which no slowly moving second component (free polysaccharide) could be demonstrated at pH 7 (Fig. 4*a*). In view of

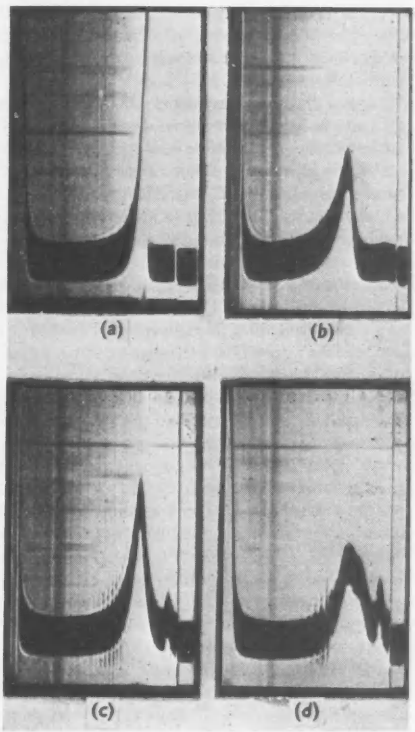

Fig. 4. Sedimentation patterns of 'Shiga' antigen fractions. Force, 30 000 *g*. Migration from right to left. (*a*) *Sp* 3/1/1 at pH 7 (phosphate:NaCl, $I=0{\cdot}2$), (*b*) *Sp* 3/1 at pH 4·5 (acetate:NaCl, $I=0{\cdot}2$), (*c*) *Sp* 3/1 at pH 8 (phosphate:NaCl, $I=0{\cdot}2$), (*d*) *Sp* 3/1 at pH 11 (glycine:NaCl). Concentrations: (*a*) 1·2% (w/v), (*b*), (*c*), and (*d*) 1·25% (w/v). Exposures (*a*), (*b*) and (*c*) at 59 min., (*d*) at 91 min.

the amount of centrifuging required to obtain a larger amount of similar material, fractions *Sp* 3/1, *Sp* 2/3 and *Sp* 3/2/1 (Table 6) were used without further treatment in most of the experiments described below.

Properties of the antigenic complex

General. The materials dissolved in water to give opalescent solutions, the degree of opalescence depending on the range of g in which the fractions were sedimented. Measured as the percentage light absorption of a 1 cm. depth of a 0·5 % solution in water, fractions *Sp* 3/2/1, *Sp* 3/1 and *Sp* 2/3 (Table 6) gave figures of 51, 61 and 68, respectively. Fraction *Sp* 3/1 had $[\alpha]_D$ 66 ± 2° in water, and the viscosity measured in acetate buffer, pH 4·5 ($I = 0{\cdot}1$, containing 0·1 M-NaCl), was 1·35 in 1·25 % solution and 1·08 in 0·5 % solution, relative to the buffer at 20°. Dissolved in borate buffers of pH's 8·5–9·5, the antigen showed marked dilatancy, the mobile solution becoming a firm gel on slight shaking, reverting to a mobile solution on standing for a few minutes.

Although the antigen after drying from the frozen state failed to dissolve in diethylene glycol it was, nevertheless, readily soluble in ethylene and trimethylene glycols. A stable, clear solution of the antigenic complex in diethylene glycol could be obtained by first dissolving the material in water and subsequently replacing the water by dialysis against diethylene glycol.

Electrophoresis. Examination of a purified antigen preparation, *Sp* 3/1/1, in the electrophoresis apparatus showed that migration was very slow, and somewhat slower at pH 4·1 than at pH 8·0; diffusion was also slow, however, and this allowed appreciable movement of the boundaries to be observed up to 48 hr. Opalescence of the antigen solutions prevented examination at higher concentrations. Exact mobility measurements were not made. Photographs obtained after 24 and 30 hr. at pH's 8·0 and 4·1 are shown in Figs. 5*a* and 5*b*, respectively. Only one component is demonstrable at both pH values.

Stability. The antigenic material essentially free from unbound specific undegraded polysaccharide showed no increase in the amount of the latter component on re-examination in the analytical ultracentrifuge after several months' storage in the freeze-dried condition at room temperature. A 1·25 % solution of the material in phosphate buffer at pH 7, after storing for 30 days at 0–2°, showed no change when re-examined in the ultracentrifuge.

Particle size. The results of examination of the material in the ultracentrifuge indicate that dissociation of the complex takes place in alkaline solution and that as the pH rises the main component travels more rapidly.

Sedimentation constants were determined using fraction *Sp* 3/1 in acetate buffer pH 4·5, $I = 0{\cdot}1$ containing 0·1 M-NaCl and phosphate buffer, pH 8·0 ($I = 0{\cdot}1$, containing 0·1 M-NaCl). The results obtained are shown in Table 7. The partial specific volume measured pycnometrically in acetate buffer, pH 4·5 ($I = 0{\cdot}1$, containing 0·1 M-NaCl) was determined at 2 % concentration and found to be 0·712. The S_{20} values plotted against concentration give curves which rise steeply towards the S_{20} axis and cannot be extrapolated, neither could sedimentation constants be obtained with solutions below 0·3 % concentration owing to rapid spreading of the boundary owing to the material being somewhat polydisperse. The curves obtained by plotting $1/S_{20}$ against concentration could be extrapolated, and the pH's 4·5 and 8 data converged to approximately 0·007 at infinite dilution. Considering the experimental errors involved and that only three points were available to draw each curve, it is thought that the sedimentation constant extrapolated to infinite dilution is probably independent of pH and lies in the region of 130–150. The mean particle weight of the material would, therefore, appear to be of the order of 1×10^7.

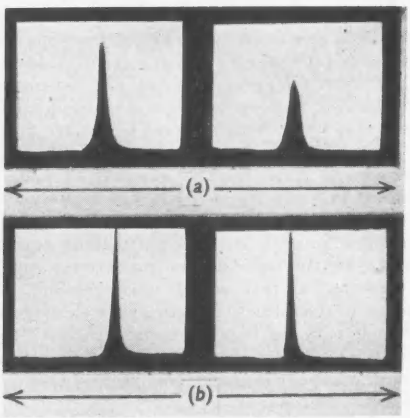

Fig. 5. Electrophoresis diagrams of 'Shiga' antigen fraction *Sp* 3/1/1, (*a*) at 0·30 % concentration, pH 8 (phosphate, $I = 0{\cdot}2$), (*b*) at 0·35 % concentration, pH 4·1 (acetate, $I = 0{\cdot}1$). Exposures at 24 hr. and 30 hr., respectively. Direction of migration shown by arrows.

Table 7. *Sedimentation constants of 'Shiga' antigen preparation (fraction* Sp 3/1)

pH	Concn. (%)	$S_{20} \times 10^{-13}$ (corr.)	$1/S_{20}$
4·5	1·25	40	0·025
4·5	0·5	84	0·012
4·5	0·3	104	0·010
8·0	1·25	29	0·034
8·0	0·5	67	0·015
8·0	0·3	91	0·011

Chemical properties. Analytical figures obtained using antigen preparation *Sp* 3/1 are: C, 44·3; H, 7·04; N, 4·56; P, 0·82; ash, 3·1; rhamnose, 22 ± 1; glucosamine (base), 14·3 ± 1 %.

Mild hydrolysis of the material in 1 % (v/v) acetic acid at 100° for 3–4 hr. in an atmosphere of N_2 gas causes the antigenic complex to separate into protein and degraded-polysaccharide components. The chemical properties of these degradation products will be given in a subsequent communication. The acid-insoluble protein is recovered by centrifugation, washing and drying from the frozen state and amounts to 20–25 % of the weight of the antigen. The degraded polysaccharide is recovered from the acetic acid supernatant fluid by precipitation with ethanol and amounts to 60–70 % of the antigen used.

Biological properties. Specific precipitation could be obtained using a sample of purified antigen (*SpM*) at a dilution of 1 in 200000. This test was carried out in a volume of 0·25 ml. using a rabbit serum prepared by the injection of six doses of purified antigen each of 10 μg.; the tubes were incubated at 37° for 2·5 hr. and the degree of precipitation observed after the tests had stood overnight at 0–2°.

The agglutination titres obtained with some of the products are shown in Table 8, together with the N and P contents of these fractions, the titres are average figures of those obtained with sera from four animals. Mixed fractions containing antigen and free specific undegraded polysaccharide, i.e. those with a low N and P content, are seen to be less antigenic than the purified materials (N, 4·4–4·6 %) and when a purified sample of the polysaccharide was injected into two rabbits in three doses amounting to a total of 100 μg., no agglutinins could be detected in the resulting sera when tested at a dilution of 1:2.

The water-insoluble fractions with a high N content were dissolved in dilute sodium carbonate and diluted with saline for injection, these materials proved to be strongly antigenic.

The toxicity of some fractions is given in Table 9; the highly purified material, *SpM*, had a LD_{50} (median lethal dose) value of about 80 μg. in the mouse. The lethal dose is seen to increase with decreasing N and P content of the materials and fraction *S5c* which is very largely undegraded polysaccharide is of relatively low toxicity.

Table 8. *Agglutination titres of sera obtained by intravenous injection of 'Shiga' antigen fractions into rabbits*

Fraction	N (%)	P (%)	Total dose (μg.)	Titre (average from four rabbits)
P2P	6·0	0·83	3	128
SpM	4·46	0·75	3	320
S4	2·7	0·50	3	48
SFa	4·6	0·88	1	20
P1/1	6·9	0·88	30	2560
S5c	2·3	0·10	30	10

Table 9. *Toxicity of antigen fractions in mice*

Fraction	N (%)	P (%)	LD_{50} (μg.) and 95 % confidence limits
SFM	4·35	0·60	52... 80...123
SFd	4·05	0·51	56... 86...131
S3	3·0	0·45	100...160...270
S5c	2·3	0·10	220...370...620

Extraction experiments with additional strains of Shigella dysenteriae (*Shiga*)

It has been found, in agreement with the finding of Morgan & Partridge (1940), that extraction of a 'Rough' strain of *Sh. dysenteriae* (Shiga) with diethylene glycol yields a small amount of material only, which, if contained in the extract obtained from a similar quantity of 'Smooth' culture would contaminate the somatic antigen preparation to a negligible extent. Thus, from 66 g. (dry wt.) of 'Rough' organisms (K. 624 R) 960 mg. of material was extracted with the diethylene glycol, but only 20 mg. of material (N, 6·3; P, 1·95 %) was recovered in the 50–60 % acetone fraction.

A highly agglutinable strain of *Sh. dysenteriae*, recently isolated from a patient, was grown in bulk, and 145 g. (dry wt.) yielded 0·4 % of its weight in the 50–60 % acetone fraction, this contained N, 5·8; P, 1·9 %. It was reported earlier (Schütze, 1944) that highly agglutinable, 'Smooth' strains of this species gave a low yield of antigen, while inagglutinable strains gave a high yield. Strain K. 624 'S' is a relatively inagglutinable strain.

Extraction with trichloroacetic acid. A batch of acetone-dried organisms weighing 25 g. was extracted with 0·25 N trichloroacetic acid for 3 hr. in the cold according to the method of Boivin, Mesrobeanu & Mesrobeanu (1933). The primary extraction product was precipitated with 6 times its volume of cold ethanol three times and the final precipitate redissolved, dialysed and freeze-dried. The product weighed 700 mg. (a yield of 2·8 %) and it contained N, 2·6; P, 0·5 %. This material gave an antiserum in the rabbit on injection of 3 μg., which agglutinated the homologous organisms to a titre of 48 (average of four animals). This titre is about that expected of a material containing 2·6 % N, if one assumes it to be a mixture of true antigen and undegraded polysaccharide (see Table 8). Examination of this material in the analytical ultracentrifuge showed this to be the case; a small, polydisperse peak could be seen due to the antigen and a large, slowly moving, sharp peak due to the polysaccharide.

A sample of this product (400 mg.) was sedimented 3 times in the preparative ultracentrifuge at 105000 *g*, each time the supernatant was carefully removed and the precipitate resuspended. The final precipitate weighed 48 mg. (12% of the whole) and contained N, 4·5; P, 1·0%. These figures are close to those found for the purified product extracted with diethylene glycol.

DISCUSSION

From a culture of the 'Smooth' form of *Sh. dysenteriae* (Shiga), a material has been obtained by extraction with diethylene glycol, acetone and ammonium sulphate fractionation, removal of phospholipid and differential high-speed centrifugation; this material induces the formation of agglutinins in the rabbit on injection of 1 μg. and kills 50% of mice at a dose of 80 μg. The antigenic material consists of a serologically specific polysaccharide associated with a protein moiety.

The removal of the last traces of free, undegraded specific polysaccharide from the antigenic material was accomplished with difficulty and the amount of centrifugation required to achieve this end did not allow the large preparations to be completely freed from the polysaccharide. The amount remaining, however, is such as to be detectable only when relatively concentrated solutions (over 1%, w/v) are examined in the analytical ultracentrifuge; its presence has not been revealed by other means. This undegraded polysaccharide, when freed from the antigenic complex, is neither antigenic in rabbits nor toxic in mice.

The phospholipid-free antigen preparations before fractionation in the preparative ultracentrifuge contained, in addition to the free undegraded polysaccharide, a material which possessed a relatively high nitrogen content (6–7%), and would not redissolve in water after separation in the centrifuge at fairly low speed (25000 *g*). The fact that this was stable in aqueous solution in the presence of the other components of the mixture before separation suggests some kind of weak, physical interaction between the different molecular species present. This 'heavy' material was a powerful antigen and appeared to be qualitatively the same as the soluble antigen, but contained relatively more protein and less of the specific polysaccharide component, and was in a state of greater aggregation.

The analytical figures of the fractions obtained in the first main centrifuge separation (Fig. 3) suggested the possibility that the antigenic material was composed of a range of molecules of varying sizes and composition, from large ones having a relatively high nitrogen content to small ones composed largely of specific polysaccharide. The final results show, however, that this conclusion is probably not correct. The antigenic complex sedimenting within the range 25000 *g*–105000 *g* shows remarkably constant analytical figures (4·5–4·6% N and 0·80–0·85% P) and the same figures were found for three fractions, *Sp* 3/1, *Sp* 2/3 and *Sp* 3/2/1, which were sedimented with different forces.

Examination in the analytical ultracentrifuge of one of the most carefully prepared antigen fractions (*Sp* 3/1) indicated that at pH 4·5 the material was in a state of greater aggregation than at higher pH values; on extrapolation to infinite dilution, however, the sedimentation constant did not differ significantly from the figure found using the data obtained from the examination at pH 8.

Dissociation of the antigenic complex in alkaline solution is evident in the ultracentrifuge patterns (Fig. 4*b–d*). At no time during the extraction and purification, however, is the material exposed to alkaline conditions. The re-examination in the ultracentrifuge of the purified product after storage in the freeze-dried condition for several months and after storage as a 1·2% (w/v) solution in phosphate buffer at pH 7·0 for 30 days failed to demonstrate that any physical changes had occurred.

The material travels slowly during electrophoresis and shows little diffusion after 48 hr., although the conjugated protein component, which will be described later, is itself highly charged and migrates rapidly.

An examination of the product obtained by extracting organisms with trichloroacetic acid according to the procedure described by Boivin *et al.* (1933) reveals that this preparation is composed of undegraded polysaccharide and antigenic material which has a nitrogen and phosphorus content similar to that found in the diethylene glycol product. The amount of antigenic material in the trichloroacetic acid extract is, however, relatively small.

SUMMARY

1. Acetone-dried cells of *Shigella dysenteriae* (Shiga) were extracted with diethylene glycol and a preparation of the somatic 'O' antigen was recovered. The material was purified by fractionation with acetone and with ammonium sulphate.

2. The phospholipid component was removed from the antigenic material and the resulting polysaccharide–protein complex fractionated by high-speed centrifugation.

3. A heavy, water-insoluble material was recovered at 25000 *g*; this was strongly antigenic.

4. Uncombined, undegraded, specific polysaccharide was present in the original diethylene glycol extracts of *Sh. dysenteriae*, and was not deposited after prolonged centrifugation at 105000 *g*.

5. The antigen fractions separating between 25000 and 105000 *g*, contained 4·5–4·6% N and

0·80–0·85 % P, were free from detectable impurities as shown by fractional solubility tests, electrophoretic examination and behaviour in the analytical ultracentrifuge at pH 7·0.

6. Specific polysaccharide dissociates progressively from the antigen at alkaline pH values.

7. Sedimentation constants at pH's 4·5 and 8 obtained for the phospholipid-free antigen and extrapolated to infinite dilution give approximately the same figure (0·007), which indicates a particle weight of the order of 1×10^7.

8. The material possesses an LD_{50} of 80 μg. in the mouse and induces formation of 'Shiga' agglutinins in rabbits on the injection intravenously of 1 μg.

We wish to thank Dr K. Smith, Lister Institute Research Student (1945–48), for much help in the earlier part of this investigation and Dr B. R. Record, of the Microbiological Research Department, Porton, for freeze-drying facilities and for electrophoretic and ultracentrifugal examinations. One of the authors (W.M.) thanks the Stiftung für Stipendien auf dem Gebiete der Chemie, Bern (1948–50) for financial support. Permission to publish that part of the work carried out at M.R.D. has been granted by the Chief Scientist, Ministry of Supply.

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Comparative Studies of 'Bile Salts'

7. BILE ACIDS OF THE COYPU, *MYOCASTOR COYPUS*

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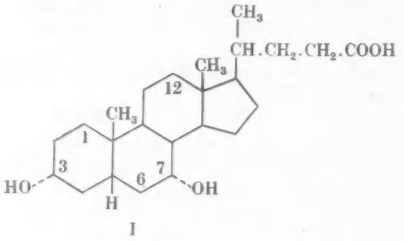
Myocastor coypus is a vegetarian rodent of South American origin which, bred for its fur (nutria), has become acclimatized in the wild state in this country and elsewhere in Europe (Laurie, 1946). Brigl & Benedict (1933) reported that the bile of this animal contained a unique bile acid, 'nutriacholic acid', conjugated with glycine; this claim was supported by Haslewood & Wootton (1950). Prof. Shimizu of Okayama University, in a letter to the present author dated February 1952, stated that Kazuno in 1946 had shown that 'nutria glycocholic acid' was a conjugate of 3-hydroxy-7-oxocholanic acid and glycine, and that coypu bile also contained ursodeoxycholic acid (3α:7β-dihydroxycholanic acid). At that time work on coypu bile in these laboratories had been continued and it was decided to attempt a comprehensive study of the bile acids. At the same time, the opportunity was to be taken of testing new methods for the purification and identification of such substances.

RESULTS

It was at once found that Kazuno's claim was well founded; 'nutriacholic acid' was identical with 3α-hydroxy-7-oxocholanic acid (III), prepared in good yield by partial oxidation of chenodeoxycholic acid (I). It was also found that condensation of this ketonic substance, as its ethyl ester, with Girard's reagent 'T' was not quantitative; nine separate Girard processes were required to extract it almost completely from the mixed ethyl esters obtained from the bile salts. In the non-ketonic material left after these experiments, esters of the following were identified: cholic (3α:7α:12α-trihydroxycholanic) acid, chenodeoxycholic (3α:7α-dihydroxycholanic) acid (I) and ursodeoxycholic (3α:7β-dihydroxycholanic) acid (II); the isolation of derivatives of these substances was greatly facilitated by the paper chromatography described below, and the ethyl esters of the last two compounds were actually first

separated and isolated in milligram quantities by this method. Cholic acid constituted less than 1% of the bile acids, and the isolated mixture of bile acids is thought to have been made up almost entirely of the three other bile acids identified, present in comparable proportions.



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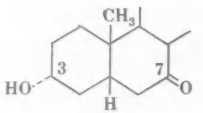
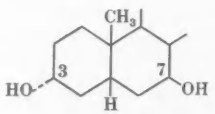


III



II

With the help of Prof. W. R. Spurrell, an experiment was carried out to try to discover whether the bile of the coypu really contained 'nutriacholic acid' produced by the liver. A drainage tube was inserted into the hepatic duct of a live animal under anaesthesia, the blood coming from the intestine was cut off and bile was collected after a period judged to be probably long enough to clear the circulating blood of products of microbial action in the gut. Such bile was found to contain a quantity of 'nutriacholic acid', and paper chromatography suggested that it also contained chenodeoxycholic and ursodeoxycholic acids.

Ethyl esters of the six possible hydroxyoxocholanic acids derived by oxidation to ketone of one or two hydroxyl groups of cholic acid (Haslewood, 1946) as well as ethyl 3α-hydroxy-7-oxocholanate and ethyl 12α-hydroxy-3-oxocholanate were tested on paper for their reaction with Kritchevsky & Kirk's (1952*a*) phosphomolybdic acid reagent; only ethyl 3α-hydroxy-7:12-dioxocholanate and 3α-hydroxy-7-oxocholanate failed to give a blue colour (compare Kritchevsky & Kirk, 1952*a*, and Sjövall, 1952).

Preparation of, and experiments with, crystalline ethyl hyodeoxycholate (ethyl 3α:6α-dihydroxycholanate) showed that this substance and ethyl ursodeoxycholate behaved in a closely similar fashion on paper chromatograms.

EXPERIMENTAL

General. Melting points were determined on a Kofler block type of apparatus and are corrected. $Al_2O_3(S)$ was 'Type H' (Peter Spence, Widnes); $Al_2O_3(N)$ was from Messrs Hopkin and Williams, neutralized as described by Shoppee (1949). Light petroleum had b.p. 40–60°, unless otherwise stated. Microanalyses (C and H) were by Drs Weiler and Strauss, Oxford.

Purification of bile acids

Hydrolysis of bile salts. Coypu bile salts (1 g., prepared as described by Haslewood & Wootton, 1950) were heated at about 110° for 6·5 hr. in a sealed metal bomb with water (5 ml.) and 5N-NaOH (5 ml.). Acidification of the diluted contents of the bomb gave a brown solid which was collected, washed and dried *in vacuo* over H_2SO_4. Yield, 5·66 g. from 9 g. of bile salts.

Esterification of bile acids. The above material (5·66 g.) was dissolved at about 23° in ethanol (60 ml.) containing H_2SO_4 (1·2 ml.). After 3 days the mixture was diluted with a solution of $NaHCO_3$ (in excess) in water. The oily neutral product was extracted with ether and the ether was washed with water, dried over Na_2SO_4 and evaporated. The residual ethyl esters weighed 5·9 g.

Girard separation of ethyl esters. The above product (5·9 g.), after being dried *in vacuo* over H_2SO_4, was heated under reflux for 1 hr. with ethanol (60 ml.) containing acetic acid (6 ml.) and Girard's reagent 'T' (2 g.). The cooled mixture was diluted, adjusted to a pH of about 7 with NaOH soln. and the 'non-ketones' were extracted with ether in the usual way. Adjustment of the aqueous residue to about 2N with HCl gave, after 16 hr. at about 25°, the 'ketones'. These were extracted with ether and the ether was washed with dilute NH_3 soln. and water, dried over Na_2SO_4 and evaporated. Acidification of the NH_3-water washings gave the 'ketonic acid' fraction (Table 1, column 3) evidently formed by hydrolysis during the above processes. This was in all cases identified by m.p. and mixed m.p. determinations as 3α-hydroxy-7-oxocholanic acid.

The 'non-ketones' from the above process were again separated as before, and this was repeated for a total of nine separations, of which the results are shown in Table 1. In this table, the working loss (column 5) was material not accounted for as the sum of the 'non-ketonic' and ketonic fractions; if there were no loss, this sum should be equal to the 'non-ketonic' fraction from the previous separation.

Table 1. *Course of Girard separations of ethylated 'bile acids' (5·9 g.) from coypu bile*

No. of Girard separations	Material separated (g.) 'Non-ketones'	'Ketones' Acidic	'Ketones' Total	Estimated working loss (g.)
1	5·091	Not collected	0·644	0·165
2	4·623	0·065	0·408	0·060
3	3·982	0·120	0·435	0·206
4	3·725	0·054	0·185	0·072
5	3·513	Nil	0·191	0·021
6	3·410	0·025	0·089	0·014
7	3·335	0·024	0·057	0·018
8	3·290	0·012	0·044	0·001
9	3·229	0·005	0·018	0·043
		Total	2·071	0·600

Table 2. *Chromatography of non-ketonic esters from coypu bile on alumina*

Esters, 3·229 g.; Al_2O_3, 70 g. in a column (28 × 2 cm.).

Fraction no.	Eluted by: Solvent	Eluted by: Vol. (ml.)	Description: Wt. (g.)	Description: Properties
I	Benzene	105	0·235	Oil
II and III	Benzene	100	0·128	Oil
IV	Benzene	100	0·004	Oil
V–IX	10, 10, 20, 30, 50% (v/v) ether/benzene	100 for each fraction	Traces	—
X	Ether	50	Trace	—
XI	5% (v/v) ethanol/ether	50	Trace	—
XII (*a*)	10% (v/v) ethanol/ether	50	Trace	—
XII	10% (v/v) ethanol/ether	20	1·526	Yellow gum
XIII	10% (v/v) ethanol/ether	25	0·844	Gum
XIV and XV	10% (v/v) ethanol/ether	100	0·090	Gum
XVI–XVIII	10% (v/v) ethanol/ether	100 for each fraction	0·060	Gum
XIX–XXII	Increasing amounts of ethanol in ether; finally ethanol	50–100 for each fraction	Few mg.	Gum
XXIII	10% (v/v) acetic acid/ethanol	150	0·220	Partially acidic yellow gum
		Total recovered	3·107	

Table 3. *Chromatography of combined ester Fractions XII and XIII, from the separation shown in Table 2, on alumina*

Esters, 2·370 g.; Al_2O_3, 25 g.

Fraction	Eluted by: Solvent	Eluted by: Vol. (ml.)	Description: Wt. (g.)	Description: Properties
A	Benzene	30	Nil	—
B	Benzene	30	1·357	Yellow gum
C	Benzene	10	0·126	Gum
D	Benzene	10	0·073	Gum
E	Benzene	20	0·075	Gum
F	Benzene	20	0·039	Gum
G	Benzene	40	0·043	Gum
H–M	Benzene	600	0·173	Gum
N	Benzene	1000	0·087	Yellow gum
O	Ether	100	0·122	Yellow gum
P	Ethanol	60	0·249	Yellow gum
	Total recovered		2·344	

Treatment of ketonic fractions. The first three ketonic fractions extracted were examined by paper chromatography as described below. Only very small amounts of material giving a colour with the spraying reagent were detected. All the ketonic esters from the separations were therefore combined and this material (1·67 g.) was hydrolysed by boiling under reflux in ethanol (20 ml.) with aqueous KOH (2 ml. of 40%, w/v) for 30 min. The clear product was cooled, diluted, and acidified with HCl. The partly crystalline acids were collected, washed with water and crystallized from ethyl acetate. Two successive crops of crystals weighed 1·19 and 0·15 g. and had melting points of 199–201° and 194–197°, respectively. This material was identical in crystalline form, m.p. and mixed m.p. with authentic 3α-hydroxy-7-oxocholanic acid prepared from chenodeoxycholic acid (p. 586). Evaporation of the final ethyl acetate mother liquors gave a gum (0·162 g.) which, after re-esterification, still showed only faint spots on paper chromatography; it was not further investigated.

Treatment of non-ketonic fractions. A solution of the final fraction (3·229 g.: Table 1) in benzene was percolated through a column (28 × 2 cm.) of Al_2O_3 (*S*, 70 g.). Fractions were eluted as shown in Table 2. Fractions XIV–XXII inclusive were examined by paper chromatography (see p. 584). Fractions XVI–XXI each gave a spot moving only a short distance from the starting line, as well as one with an R_F of about 0·5 (e.g. Fig. 1 *A*). The former spot was shown to run at the same rate as an ethyl cholate and the latter as an ethyl chenodeoxycholate standard. Fractions XVII and XVIII became partially crystalline on standing with light petroleum; the gummy crystals were recrystallized from light petroleum/benzene. Crops from XVII and XVIII had melting points 156–158° and 159–162°, respectively, not depressed by admixture with ethyl cholate. The crystals gave a blue colour in the Hammarsten HCl test. No ethyl cholate spot showed on paper chromatography of fractions XIV, XV or XXII; hence the maximum amount of ethyl cholate which could have been present in the non-ketones was about 100 × 0·06/3·229, i.e. about 1·9%, and was probably less than half this quantity. There was thus considerably less than 1% of cholic acid in the coypu bile acids examined. Fractions I–III and also XXII were not further examined.

The main fractions, i.e. XII and XIII, were combined, dissolved in benzene and separated on Al_2O_3 (*N*, 25 g.) on a column, with the results shown in Table 3. Fractions *A–O* were each examined by paper chromatography and all showed spots corresponding to ethyl chenodeoxycholate. However, in certain fractions, notably *D*, *E* and *F*, a second spot was seen, running at about half the rate of ethyl chenodeoxycholate (Fig. 1, *B*, *C*). Fractions *D–F* were combined, hydrolysed in the usual way and an attempt was made to separate the acids by partition chromatography as described by Bergström & Sjövall (1951). This was unsuccessful, but J. Sjövall subsequently showed, in the author's laboratory, that a separation was possible with a 10% (v/v) heptane:chloroform/55% (v/v) methanol:water system (see Sjövall, 1952). The esters were separated by paper chromatography; that giving the upper spot was ethyl chenodeoxycholate and that giving the lower spot was ethyl ursodeoxycholate (see pp. 584, 585).

Fraction *B* (Table 3) was examined by infrared spectroscopy by Mr H. Wiggins, in Dr I. D. P. Wootton's laboratory (Wootton, 1953): it was reported to consist of ethyl chenodeoxycholate with another component, not ethyl deoxycholate.

A solution of fraction *P* (0·249 g.) in ether was washed successively with dilute HCl, water, dilute NH_3 soln. and water. The ether was dried over Na_2SO_4 and evaporated, and the residue (0·244 g.) was examined by infrared spectroscopy as above and by paper chromatography. The examination by infrared spectroscopy suggested that it consisted very largely of ethyl chenodeoxycholate and only one spot, corresponding to this, was seen on the paper. However, it is felt that some substance(s) of a different type may have been present. The impression was gained that all the fractions from Al_2O_3 (Table 3) contained ethyl chenodeoxycholate, but that ethyl ursodeoxycholate was present also in considerable amounts in most fractions. It constituted the greater proportion in fractions *D–F* (see p. 585).

Paper chromatography of bile acids and their esters

Sjövall (1952), Kritchevsky & Kirk (1952*b*) and Beyreder & Rettenbacher-Däubner (1953) have described methods different from the following.

General methods. Use was made of the solvent systems of Bush (1952) and of the 10% (w/v) phosphomolybdic acid-in-ethanol, spraying reagent of Kritchevsky & Kirk (1952*a*). Strips of Whatman 3MM paper were ruled with the starting line at 8–10 cm. from one end. When the ethanolic solutions of substances to be run had been applied at the starting line (0·5 cm. diam. spots) and allowed to dry, the paper was hung in a glass tank so that the starting line was at the bottom. The tank was now tilted and the moving phase was added so as to be contained in the lower part of the tank; the paper was prevented from reaching this phase by a beaker containing the stationary phase. This stationary phase was allowed to run up a narrow strip or strips of filter paper. The tank was sealed with (*a*) a cloth moistened with stationary phase, (*b*) a cellophan cover and (*c*) a rubber cover, all tightly held by rubber bands. At least 16 hr. were allowed for equilibration, after which the tank was carefully tilted so as to allow the moving phase to reach the loaded paper. When the solvent front had reached a distance of about 20–22 cm. above the starting line, the paper was removed, dried in air and at about 90°, sprayed and heated for about 2 min. at 100° to bring up the blue spots. As the temperature was not controlled within 20–25°, R_F values were not reliable (Bush, 1952), and standard solutions (1–10 mg./ml. in ethanol) on the same paper were used to identify spots. The only difficulty encountered was that, when there had been wide variations in temperature, the stationary phase sometimes condensed on the tank walls and then ran on to the paper when the tank was re-tilted after equilibration. If this caused serious trouble, the experiment could be stopped before the solvent front had reached the starting line, the paper briefly dried in air and the whole re-assembled with fresh samples of the phases. Equilibration in these circumstances only required about 2 hr.

Results. The following could easily be separated, using the systems of Bush (1952) mentioned in brackets: cholic, deoxycholic or chenodeoxycholic, hyodeoxycholic and lithocholic acids (*B* 5); ethyl cholate, trihydroxycoprostanate (Haslewood, 1952), deoxycholate or chenodeoxycholate, and hyodeoxycholate (*A*). Deoxycholic and chenodeoxycholic acids and their methyl or ethyl esters were not separable by these methods (see also Sjövall, 1952). Opportunity was taken to test the reactions of the following, as spots (containing about 30 μg.) on paper: the six possible ethyl hydroxyoxocholanates derived by oxidation to ketone of one or two hydroxyl groups in ethyl cholate (Haslewood, 1946); ethyl 3α-hydroxy-7-oxocholanate and ethyl 12α-hydroxy-3-oxocholanate. A blue colour was given by all except ethyl 3α-hydroxy-7:12-dioxocholanate and ethyl 3α-hydroxy-7-oxocholanate. 3α-Hydroxy-7:12-dioxo-, 3α-7α-dihydroxy-12-oxo- and 3α-hydroxy-7-oxocholanic acids were tested by Sjövall (1952); the present results agree with his for the parent acids. Lithocholic acid and its ethyl ester also gave a rather faint colour, not detected by Sjövall (1952). Sjövall reported that 3α-hydroxy-12-oxocholanic acid gave no colour with the reagent.

Separation of ethyl chenodeoxycholate and ethyl ursodeoxycholate on paper. Pieces of Whatman 3MM paper about 30 × 22 cm. were ruled across the longer axis to make a starting line 6·5 cm. from one end. This line (22 cm. long) was marked with dots at 0·5 cm. intervals. A solution (66 mg. in 3·3 ml. of ethanol) of the mixed ethyl esters obtained in fractions *D–F* (Table 3) was allowed to fall, from a graduated pipette drawn out into a fine capillary, on to these dots so as to make a confluent row of spots. The amount on the paper (about 5 mg.) was known by reading the pipette. A large battery jar served as a tank for each paper and the separations were carried out exactly as described above, with Bush's system *A*. When the papers had been dried, strips 0·5 cm. in width were cut from a long edge and sprayed; the appearance is shown in Fig. 2. These sprayed strips were laid beside the rest of the paper, as they had been cut, and the unsprayed paper was ruled across so that the transverse zones (shown by the strips) occupied by the two separated substances could be cut out separately. Fourteen such separations were done involving almost all the material (66 mg.). The combined 'upper' and 'lower' zones cut from the papers as described above were extracted separately in a Soxhlet apparatus with methanol. Evaporation of the methanol left, in each case, a yellowish gum, which was further purified by passage in benzene through a column containing Al_2O_3 (*N*, 0·5 g.). The recovery was: from the combined upper zones—22 mg.; from the combined lower zones—40 mg.; total, 62 mg. It was calculated that unavoidable losses amounted to about 4 mg.; hence recovery was quantitative. Mr Wiggins, by infrared spectroscopy, found that the material from the upper zone was ethyl chenodeoxycholate and from the lower zone was a hitherto unexamined substance, converted on chromic oxidation into ethyl dehydrochenodeoxycholate (3:7-dioxocholanate). On paper chromatography, material from each zone gave only a single spot, in their expected positions (Fig. 3). The lower zone material could not be ethyl hyodeoxycholate (spectroscopic evidence) although the spot on paper from it (Fig. 4) corresponded to this substance. It was hydrolysed, and the acid obtained crystallized readily from dilute ethanol on seeding with authentic ursodeoxycholic acid, the gift of Prof. T. Shimizu. Shimizu's ursodeoxycholic acid melted at 202–204°, the acid from the lower zone at 203–204·5° and the mixture at 202–204°.

Material from the upper zone (20 mg.) was dissolved in acetic acid (0·5 ml.) with acetic anhydride (0·15 ml.). 6N-$HClO_4$ (1 drop) was added and, after 25 min., the mixture was diluted and extracted with ether. The ether was washed with water, NH_3 soln., and water, then dried over Na_2SO_4

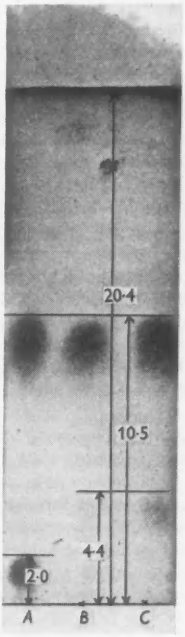


Fig. 1.

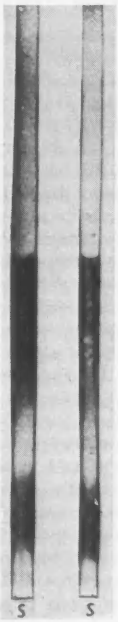


Fig. 2.

Fig. 3.

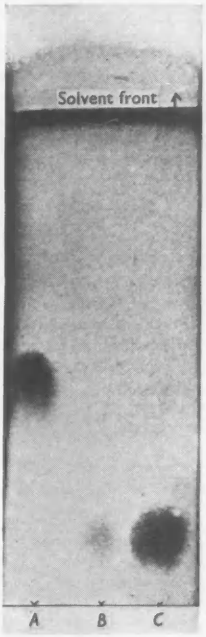


Fig. 4.

Fig. 1. Paper chromatogram of fractions separated on Al_2O_3 of bile acid esters from the coypu. *ABC*, starting line. *A*, fraction XVI (Table 2); *B* and *C*, mixed fractions *D–F* (Table 3). Solvent system *A* (Bush, 1952).

Fig. 2. Strips cut from sheets of paper used to separate mixed fractions *D–F* (Table 3) from bile acid esters from coypu bile. *S*, *S*, portions of starting line. Upper and lower zones (dark) show positions of separated substances on whole paper.

Fig. 3. Paper chromatogram of substances separated into zones as shown in Fig. 2 and described in text. *AB*, starting line. *A*, from upper zone (ethyl chenodeoxycholate); *B*, from lower zone (ethyl ursodeoxycholate). Solvent system *A* (Bush, 1952).

Fig. 4. Paper chromatogram of purified ethyl esters of bile acids. *ABC*, starting line. *A*, ethyl chenodeoxycholate; *B*, ethyl hyodeoxycholate; *C*, ethyl ursodeoxycholate. Solvent system *A* (Bush, 1952).

and evaporated. The residue, in light petroleum, was boiled with charcoal and the filtered solution evaporated. The colourless residue crystallized on standing at about 5° with a little light petroleum, giving large, colourless crystals of m.p. 102–106°, not depressed by ethyl diacetylchenodeoxycholate (m.p. 104–106°).

It was concluded that the combined fractions *D–F* (186 mg., Table 3) contained ethyl chenodeoxycholate (about 62 mg.) and ethyl ursodeoxycholate (about 124 mg.). Ethyl ursodeoxycholate and ethyl hyodeoxycholate ran at virtually the same rate on paper (Fig. 4) and both substances gave a sluggish and rather faint reaction with the spraying reagent.

Collection and treatment of bile from a live coypu

Collection. A coypu about 5 years old (senile) and weighing about 9 lb., the gift of Mr G. Iles, was anaesthetized with Nembutal. The abdomen was opened and the gall bladder was tied off, emptied of bile with a syringe and removed. A cannula was put into the hepatic duct and the portal vein was tied. Bile flowing from the liver (at the rate of 4–6 ml./hr.) was collected during 3 hr., after which the animal died. The operative procedures were carried out by Prof. W. R. Spurrell.

Treatment of bile. The gall-bladder bile (16·5 ml.) was diluted with ethanol (50 ml.) and the filtered solution was

evaporated to give the bile salts (0·638 g.; approx. 3·9% (w/v) of the bile). The first hour's collection of liver bile was discarded and the bile (11 ml.) collected during the second and third hours was treated as above to obtain the bile salts (0·224 g.; approx. 2·2% (w/v) of the bile). Each sample of bile salts was hydrolysed and the resulting bile acids were isolated and esterified as described above. Yields: gall-bladder bile esters, 0·34 g.; liver bile esters, 0·056 g. Each sample of esters was separated once into ketones and 'non-ketones' with Girard's reagent 'T' as described above. The yields of ketones for a single separation cannot be considered significant (see p. 581), but each ketonic fraction gave, after hydrolysis, on crystallization from ethyl acetate and then from dilute ethanol, a high yield of colourless leaflets. Samples from gall-bladder and liver bile both had m.p. 195–197°, unchanged by admixture with an authentic sample of 3α-hydroxy-7-oxocholanic acid.

The 'non-ketones' from the liver bile (0·042 g.) were dissolved in benzene and run through Al_2O_3 (*N*, 0·5 g.) in a column. Benzene (60 ml.) eluted a colourless gum (0·027 g.) which was dissolved in ethanol (0·2 ml.). When the solution was examined by paper chromatography with Bush's system *A*, spots were obtained corresponding to those given by ethyl chenodeoxycholate and ethyl ursodeoxycholate.

Preparation of bile-acid derivatives

3α-Hydroxy-7-oxocholanic acid (*III*). Chenodeoxycholic acid (0·1 g.) was dissolved by warming with acetic acid (1 ml.) and sodium acetate ($CH_3CO_2Na.3H_2O$; 0·2 g.). To the solution at about 20° was added a solution of potassium chromate (K_2CrO_4, 31·7 g./100 ml.; 0·15 ml.). The mixture was shaken at intervals until the chromate had dissolved (about 30 min.) and was then left with occasional shaking at about 20° for 6 days, during which time crystals separated. Dilution with water gave a crystalline solid which was collected, washed and recrystallized from ethyl acetate, from which the above acid separated as colourless prisms (0·06 g.; 60%) of m.p. 195–197°. *Ethyl 3α-hydroxy-7-oxocholanate*, prepared in the usual way and crystallized from a small quantity of light petroleum, formed white needles of m.p. 61–63°. (Found: C, 74·2; H, 9·9. $C_{26}H_{42}O_4$ requires C, 74·6; H, 10·1%.) *Ethyl 3α-acetoxy-7-oxocholanate*, prepared by acetylation in the usual way of the above ester, formed colourless needles from light petroleum and white leaflets from dilute ethanol; it had m.p. 120–121°. (Found: C, 73·2; H, 9·5. $C_{28}H_{44}O_5$ requires C, 73·0; H, 9·6%.) This substance closely resembled ethyl 3α:7α-diacetoxycholanate (ethyl diacetyl chenodeoxycholate) in appearance and behaviour, and crystals of either compound could be used successfully to 'seed' preparations and solutions of the other to induce crystallization.

Ethyl hyodeoxycholate. A solution of hyodeoxycholic acid (0·1 g.) in ethanol (2 ml.) containing H_2SO_4 (0·04 ml.) was left at about 20° for 4 days. The diluted mixture was stirred with $NaHCO_3$ (in excess) and the oily product extracted with ether. The ether was washed with water, dried over Na_2SO_4 and evaporated. The gummy residue was purified by passage in benzene through Al_2O_3 (*N*, 1 g.) and the product left after evaporation of the benzene eluate crystallized on standing with light petroleum. *Ethyl hyodeoxycholate* (*ethyl 3α:6α-dihydroxycholanate*) crystallized from light petroleum/benzene as long, white needles of m.p. 114–116°. (Found: C, 74·2; H, 10·4; $C_{26}H_{44}O_4$ requires C, 74·3; H, 10·5%.)

Ethyl ursodeoxycholate (ethyl 3α:7β-dihydroxycholanate). This was prepared in a similar fashion, but showed no tendency to crystallize.

DISCUSSION

Biological. Coypu bile has been shown to contain chiefly chenodeoxycholic acid and substances (II and III) which may be considered to be derived from it. In this respect, it resembles the bile of the guinea pig, which Imai (1937) has shown to contain chenodeoxycholic and 3α-hydroxy-7-oxocholanic acids. In both species the conjugation is almost entirely with glycine, although in the coypu a small quantity of bile acid conjugated with taurine probably is present (Haslewood & Sjövall, 1954). Other species showing almost entirely glycine conjugation are the rabbit and pig; in neither of these cases can the bile acids be considered to be derived from chenodeoxycholic acid.

The result of the experiment now reported with the live coypu makes it seem probable that the ketonic acid in the bile of this species is a true product of the liver and not a substance formed by micro-organisms in the intestine. This seems to be an important point, especially as ketonic acids are frequently cited as intermediates in bile acid metabolism. It would be interesting to know whether preparations of coypu liver can convert chenodeoxycholic acid or its glycine conjugate into the 7-oxo derivative. Hoehn, Schmidt & Hughes (1944) showed that oxidation (to ketone) at position 7 could be readily brought about in cholic acid by a strain of *Acaligenes faecalis* and, of course, analogous oxidations both by micro-organisms and by tissue preparations are well known in steroids other than bile acids. There is therefore nothing extraordinary in the idea that a ketonic acid should be a major constituent of the bile acids in a species, although this seems to be a somewhat unusual occurrence.

The finding of ursodeoxycholic acid in the coypu destroys the view that this substance is characteristic of the bile of bears. Earlier history of work on this acid was discussed by Haslewood & Wootton (1950) who concluded that bear's bile contained cholic acid as its chief constituent. The results of paper chromatography on the liver bile esters from the coypu suggested that ursodeoxycholic acid may be made by the liver itself in this species.

One result of Kazuno's and the present investigation is to remove all grounds for the use of the name 'nutriacholic acid', which is neither an acid unique in coypu bile nor a cholic (trihydroxycholanic) acid. Coypu bile is in fact 'unique' in the same sense as guinea pig bile and the relationship

may possibly indicate more general biological affinities.

It is tempting to suppose that the small amount of cholic acid now shown to be present in *Myocastor coypus* represents a vestigial 'memory' of a previous time in which cholic was (as in most mammals today) the chief bile acid; this view implies that it is more recent evolution which has produced the 'glyco-dihydroxy' type of bile salt discussed above.

Chemical. The present work puts beyond doubt the superiority of methods of partition chromatography over the hitherto usual separations on alumina for investigations of mixtures of bile acids. Complete separation of the dihydroxy esters clearly would not have been possible without partition methods. The isolation after paper chromatography described here was easy to carry out and could be applied in all instances where more than one spot can be obtained. Separation on alumina remains a valuable method of handling material, especially in larger quantities, for preliminary purification.

A working hypothesis which will explain the (at present) known results of testing ketonic bile acids and esters with the phosphomolybdic acid reagent is the following. In cholanic acid derivatives containing oxygen at positions 3, 7, 12 or any one or two of these, at least one hydroxyl group in the α position is needed to reduce the reagent; an α-hydroxyl group at C-3 only will not give a reaction in the presence of carbonyl groups at C-7, C-12 or both positions. An α-hydroxyl at C-7 or C-12 will reduce the reagent irrespective of carbonyl groups at one or both of the remaining positions considered. Such rules, if confirmed, may be of value in the study of constitutional problems; they are clearly capable of considerable amplification.

The resistance of ethyl 3α-hydroxy-7-oxocholanate to reaction with Girard's reagent 'T' was not altogether unexpected, for Hoehn *et al.* (1944) had already noticed that 3α:12α-dihydroxy-7-oxocholanic acid 'does not form a Girard hydrazone'.

SUMMARY

1. An investigation of the chemical nature of the bile acids of the coypu, *Myocastor coypus*, has been carried out. In agreement with Kazuno, it has been found that the 'nutriacholic acid' of Brigl & Benedict (1933) is 3α-hydroxy-7-oxocholanic acid, and that coypu bile contains ursodeoxycholic (3α:7β-dihydroxycholanic) acid. Chenodeoxycholic (3α:7α-dihydroxycholanic) and cholic (3α:7α:12α-trihydroxycholanic) acids have now also been found in the bile of this species. The name 'nutriacholic acid' should no longer be used.

2. Examination of bile collected from a living coypu suggested that 3α-hydroxy-7-oxocholanic and ursodeoxycholic acids may be primary products of the liver and not artifacts absorbed from the intestine.

3. Some biological implications of the nature of coypu bile have been briefly discussed.

4. Methods of paper chromatography have been used to trace and to isolate the above bile acids; such methods were more effective than chromatography on alumina alone.

5. Ethyl 3α-hydroxy-7-oxocholanate was resistant to reaction with Girard's reagent 'T', and nine extractions were necessary to remove it effectively from the mixture of ethylated bile acids.

6. The ethyl esters of eight hydroxy-keto bile acids, substituted at two of, or all, the positions 3, 7 and 12 of the cholanic acid molecule have been tested, as spots on paper, with the phosphomolybdic acid reagent of Kritchevsky & Kirk (1952*a*). The results, together with those previously reported by other workers, can be explained by a simple working hypothesis.

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On the Bromide Test of Permeability of the Barrier between Blood and Cerebrospinal Fluid—an Assessment

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The publication of Walter's monograph (1929) on the blood/cerebrospinal-fluid barrier was followed by the widespread use of the bromide test in diseased conditions of the brain and spinal cord. The bromide test consists in the administration of bromide to the patient; after allowing 24 hr. for equilibration with the cerebrospinal fluid (c.s.f.), the content of bromide in the serum and c.s.f. is determined. The permeability of the barrier is then judged by the magnitude of the ratio (bromide/unit vol. serum)/(bromide/unit vol. c.s.f.). Walter found normal control ratios to lie in the range 2·90–3·30 (mean 3·10). In pathological states causing the greatest increase in permeability (syphilitic or tuberculous meningitis) the ratio was near 2·0, although a few values as low as 1·50 have been recorded. On the other hand, ratios over 3·50 were found in a number of conditions.

Walter emphasized certain desirable features of the bromide test. Bromide is excreted very slowly, so the blood level remains relatively constant over long periods (days or weeks), permitting adequate time for the establishment of equilibrium between blood and c.s.f., and making possible several tests without further bromide administration. Bromide does not enter metabolism and might thus be expected to qualify as a test substance for permeability. It is neither bound by, nor adsorbed to, protein. It can be determined with adequate precision at low levels in serum or c.s.f. It is neither disliked by, nor toxic to, patients, and at the levels used is unlikely to affect barrier permeability. Its normal distribution between serum and c.s.f. gives a ratio well removed from unity so that decreases or increases in permeability are readily recognized.

A fairly extensive study of neurological conditions with the bromide test has enabled us to examine a number of points relevant to the test which have received little or no attention. From the beginning we regarded the auric chloride method of bromide determination (Walter, 1919; Malamud, Mullins & Brown, 1933; Katzenelbogen & Czarski, 1934) with some scepticism. Since several workers have questioned its reliability (e.g. Lovell & Brown, 1934; Tod, 1933; Mishkis, Ritchie & Hastings, 1933; Fremont-Smith, Dailey & Sloan, 1935; Gray & Moore, 1942) a method based upon iodometric titration (Hunter, 1953), and regarded as more accurate, has been used throughout. With this method, however, the ratios obtained were markedly lower than those commonly reported, our mean for nearly normal controls being 2·60 (s.d. 0·30). This necessitated the examination of sera and c.s.f. by both methods, and it was found that there is commonly present in human serum a substance that reacts like bromide with auric chloride solutions to give a brown colour. With c.s.f. the two methods give results in near agreement but with serum the result by the auric chloride method is commonly too high, and an erroneously high bromide distribution ratio is obtained.

As bromide tests are carried out under ordinary hospital working conditions, the technique for collecting blood should be as simple as possible. The use of whole blood overcomes analytical uncertainties that may arise from uneven distribution of ions between cells and plasma, but it necessitates an anticoagulant, and is less suitable than plasma or serum for studies on the balance of electrolytes with other body fluids. Therefore we decided to use serum for bromide analysis; this raised the question whether there was a 'shift' of bromide, like that of chloride, with changing pH and degree of oxygenation of haemoglobin. This seemed probable from the little information available, especially on human blood. The normal distribution of administered bromide between plasma and corpuscles also seemed to call for further observation since false bromide distribution ratios might arise through alteration of the serum bromide after taking the blood. Some of our blood samples might be kept several days before the serum was separated from the clot.

In the determination of serum/c.s.f. bromide ratios Walter states that blood and c.s.f. were taken 24 hr. after the last oral dose of bromide. Yet there is little information in the literature as to how long administered bromide takes to reach equilibrium with the c.s.f. Nor is there adequate evidence that

the ratio is consistent from day to day in the same person in similar conditions. This paper describes experiments designed to assess the general reliability of the Walter test.

The convention that Br_s = bromide in serum and $(Br)_s$ = bromide in serum water has been followed. Other subscripts used are p = plasma, c = cells, and c.s.f. = cerebrospinal fluid.

EXPERIMENTAL

Determination of bromide. The iodometric titration method of Hunter (1953) and the colorimetric auric chloride method as modified by Katzenelbogen & Czarski (1934) were used.

Comparison of results from iodometric and auric chloride methods

Table 1 shows bromide values obtained on sera and c.s.f. and the resulting ratios, by the two methods. The colours obtained with auric chloride were read in a photoelectric colorimeter at 450 mμ., in the region of maximum absorption for auric bromide (which is the substance that gives the brown colour), and also at 500 mμ., as likely to accord better with readings in a visual colorimeter. It will be seen that there is fair concordance with both the titrimetric and the colorimetric method, at either wavelength, for c.s.f. With serum, however, the values obtained colorimetrically are higher and often much higher, especially at 500 mμ., than the corresponding titrimetric values. The first four cases listed in Table 1 have about normal permeabilities with a mean ratio, by the titration method of 2·67 or by the colorimetric method at 450 mμ. of 2·79 and at 500 mμ. of 3·11, a value the same as the Walter mean. The lower ratios are similarly increased in the three cases of tuberculous meningitis. Most striking perhaps are the values on the sera of the five individuals not treated with bromide; their sera contained usually less than 1 mg. Br/100 ml. according to the iodometric method but much more according to the colorimetric method. It may be noted that Gray & Moore (1942), in an examination of blood from seventy-six subjects who had received no bromide, obtained by the auric chloride method results indicating 16–53 mg. Br/100 ml. in nine of these cases, although no bromide was detected by other tests.

It seems that human sera, normal and pathological, contain a substance (or substances) which is not bromide but which produces, like bromide, a brown colour in auric chloride solution and that this substance is responsible for the high mean value of the ratio $Br_s/Br_{c.s.f.}$ obtained when auric chloride is used to determine bromide in serum. The nature of the interfering substance in serum is unknown; a variety of substances in dilute solution, such as iodide, sulphide, thiosulphate, guaiacol, increase the brown colour of auric chloride solutions under the conditions of the test, whereas small amounts of uric acid or ascorbic acid nearly completely discharge the colour with formation of metallic gold. On such grounds alone it is not surprising that the use of this reagent leads to uncertain results in blood serum.

Rate of equilibration of bromide between plasma and corpuscles

The experiments recorded in Table 2 show that equilibration of bromide between plasma and corpuscles is established within 5 min. of addition of bromide *in vitro* and within 30 min. *in vivo*.

Table 1. *Bromide distribution ratios in serum and cerebrospinal fluid*

Bromide was determined by iodometric titration and by the auric chloride method. In the latter the absorption was measured at 450 and at 500 mμ.

Patient		Iodometric titration method			Auric chloride method, measurement at 450 mμ.			Auric chloride method, measurement at 500 mμ.		
		Bromide in			Bromide in			Bromide in		
No.	Diagnosis	Serum (mg./100 ml.)	C.s.f. (mg./100 ml.)	$\frac{Br_s}{Br_{c.s.f.}}$	Serum (mg./100 ml.)	C.s.f. (mg./100 ml.)	$\frac{Br_s}{Br_{c.s.f.}}$	Serum (mg./100 ml.)	C.s.f. (mg./100 ml.)	$\frac{Br_s}{Br_{c.s.f.}}$
94S	Palsy	18·8	7·2	2·61	16·2	7·0	2·31	22·3	7·2	3·10
97S	Pain	18·8	7·0	2·69	18·3	6·6	2·78	23·0	7·3	3·15
98S	Pain	20·7	7·8	2·65	23·8	8·4	2·81	26·1	8·2	3·18
99S	Disseminated sclerosis	18·3	6·7	2·73	22·1	6·8	3·27	25·0	8·3	3·00
128X	Tuberculous meningitis (treated cases)	22·8	15·9	1·43	24·6	14·2	1·73	29·7	15·0	1·98
95X		26·4	15·7	1·68	28·2	15·7	1·79	36·0	15·3	2·35
113X		27·6	13·3	2·07	36·0	14·1	2·55	40·8	14·1	2·90
—	Normal	0·9	—	—	1·8	—	—	5·4	—	—
120X	Meningitis	0·5	—	—	3·8	—	—	4·5	—	—
141X		0·4	—	—	4·7	—	—	8·1	—	—
127X		1·1	—	—	6·3	—	—	18·0	—	—
134X	Transverse myelitis	0·9	—	—	5·7	—	—	17·1	—	—

Table 2. *Rate of equilibration of bromide with blood* in vitro *and* in vivo

In vitro. NaBr (as 0·6 ml. of isotonic glucose solution containing 5·38 mg. Br) was added to 20 ml. heparinized blood. Samples (3 ml.) were taken for analysis at intervals. The packed cell volume was 48·5%.

In vivo. Heparinized blood was obtained after intravenous injection of NaBr.

Time after addition of NaBr (min.)	Bromide (mg./100 ml.) Plasma (*a*)	Cells (*b*)	Whole blood Found	Whole blood Calc. from *a* and *b*	$\frac{Br_c}{Br_p}$
In vitro					
5	32·1	—	—	—	—
10	32·6	—	—	—	—
15	33·0	—	—	—	—
30	32·5	—	—	—	—
60	32·6	—	—	—	—
17 hr.	32·5	19·3	26·6	26·1	—
In vivo					
30	27·8	15·4	—	—	0·55
26 hr.	23·7	13·3	—	—	0·56

Table 3. *Effect of* pH *on equilibration of blood with bromide added* in vitro *and* in vivo

In vitro. NaBr in isotonic glucose solution was added to fresh, heparinized human blood. The mixed blood was divided into four parts. One part was untreated; N-NaOH or N acetic acid in bromide-free saline was added to the others to give the range of plasma pH shown. After 15 min. the bloods were centrifuged and bromide determined. (The top layer of cells containing 'buffy coat' was removed and discarded.) It was assumed that plasma and cells contained 93 and 70% of water, respectively (Maizels, 1936; Harris & Maizels, 1952).

In vivo. Heparinized blood was taken from two patients who had previously received bromide.

Blood no.	Bromide added	Bromide (mg./100 ml.) Plasma	Cells	Plasma water	Cell water	$\frac{(Br)_c}{(Br)_p}$	Plasma pH at 20°
1 (86S)	*In vitro*	42·0	24·6	45·2	35·1	0·77	8·0
2		40·2	26·4	43·2	37·7	0·87	7·8
3		36·0	28·4	38·7	40·6	1·05	7·5
4		35·1	28·2	37·7	40·2	1·06	7·2
1 (76S)	*In vivo*	18·1	7·6	19·4	10·9	0·56	7·98
2		17·7	9·5	19·0	13·6	0·72	7·57
3		15·8	9·8	17·0	14·0	0·82	7·15
4		15·2	9·9	16·3	14·1	0·86	7·00
1 (75S)	*In vivo*	11·8	5·8	12·7	8·3	0·65	8·00
2		11·7	7·0	12·6	10·0	0·79	7·56
3		10·0	7·2	10·8	10·3	0·96	7·18

Effect of pH *on distribution of bromide between plasma and cells*

Table 3 shows that the ratios of bromide in cell water to bromide in plasma water, $(Br)_c/(Br)_p$, vary inversely as the pH. It may be noted that the change in plasma water through addition of solutions of base and acid is less than 1% and has been neglected in the calculation.

Bromide distribution in blood collected from hospital patients

Venous blood was collected in a syringe and mixed directly with heparin in a corked test tube. The tube, with cork in place to prevent loss of carbon dioxide, was centrifuged. From twenty-four observations on nineteen patients suffering from a variety of neurological conditions and including only one febrile case, the ratio $(Br)_c/(Br)_p$ was found to be 0·84 (S.D. 0·08). The observations are not given separately as there seemed to be no relationship between $(Br)_c/(Br)_p$ and sex, age, pathological condition, or plasma pH.

Shift of bromide in stored blood

It seemed possible that on storage of blood a change in the ratio $(Br)_c/(Br)_p$ might occur even if there was no change in pH. Accordingly the bromide level was followed in heparinized blood kept at room temperature and in clotted blood stored in a refrigerator for several days. The packed cell

Table 4. *Effect of storage on plasma or serum bromide*

Heparinized blood was stored at 20° and clotted blood at 4°. Plasma and cell water were assumed to be 93 and 70%, respectively.

No.	Time of storage of blood (days hr.)	pH of plasma or serum at 20°	Packed cell volume	Bromide (mg./100 ml.) Plasma or serum	Cells	Plasma when $(Br)_c/(Br)_p = 1$	Maximum fall in plasma or serum bromide (%)
			Heparinized blood				
72	0·5	—	37	24·0	13·3	—	—
	4	—	—	21·8	—	—	—
	8	—	—	21·4	—	22·0	10·8
74	0·5	—	42	20·3	12·5	—	—
	4	—	—	20·1	—	—	—
	2	7·58	—	19·1	—	18·8	6
77	17	7·56	41	20·4	13·7	—	—
	2	7·56	—	19·9	—	19·6	2·5
81	1	7·56	44	17·1	12·0	—	—
	4	7·40	—	16·5	—	16·7	3·5
			Clotted blood				
83	4	7·72	—	29·4	—	—	—
	2	7·80	—	29·3	—	—	—
	4	7·60	—	28·3	—	—	3·7
87	1	7·63	—	13·8	—	—	—
	2	7·61	—	13·6	—	—	—
	6	7·64	—	13·3	—	—	3·6
90	2	7·50	—	20·3	—	—	—
	3	7·54	—	19·8	—	—	—
	6	7·71	—	20·3	—	—	—
	12	7·24	—	19·7	—	—	3·0
91	2	7·57	—	18·9	—	—	—
	4	7·57	—	18·9	—	—	—
	10	7·52	—	18·1	—	—	4·2

volume of the former was determined and the bromide level expected in the plasma when the anion becomes evenly distributed in cell and plasma water was calculated. The results from several experiments are given in Table 4 which shows that all plasma or serum values tend to fall towards the calculated level.

DISCUSSION

Equilibration of bromide with blood

It is apparent from Table 2 that bromide added *in vitro* or *in vivo* to human blood rapidly reaches equilbrium with the cells. This finding is in accord with the observations of Hastings & Van Dyke (1931) on dog blood. Table 3 shows that whether bromide is added *in vitro* or *in vivo* to blood its distribution between the cells and plasma varies, like chloride, with pH. This finding is also in accord with those of Hastings & Van Dyke (1931) and of Hastings, Harkins & Liu (1932), when very large amounts of bromide were given to dogs. If the ratios in Table 3 are plotted against pH they have about the same slope, with a shift of 0·3 pH unit causing a change of about 10% in the ratio. The chloride shift with pH as found by Harris & Maizels (1952) has a similar magnitude. At normal blood pH the magnitude of the chloride ratio, $(Cl)_c/(Cl)_p$ is by general consent (see Owen & Power, 1953) close to 0·70 for man. In nine female epileptic patients, Notkin, Garcia & Killian (1933) found a mean bromide ratio of 0·80 from bloods collected without precautions to exclude air. Their corresponding chloride ratio was 0·82. In four patients with bromide intoxication, Mason (1936) found a mean chloride ratio of 0·73 and a corresponding bromide ratio of 0·80. In dogs, Weir & Hastings (1939) found a chloride ratio of 0·72 and a bromide ratio of 0·76 (ten observations). Our bromide ratio, from twenty-four observations on nineteen persons, is 0·84 (S.D. 0·08). This is significantly higher than the few values previously reported for human blood and serves to emphasize that bromide displaces more than its chloride equivalent from erythrocytes.

Hastings and his associates used venous blood collected under anaerobic conditions. Our blood samples were collected with minimum exposure to air, but not with strict anaerobic technique, with some consequent oxidation of haemoglobin and loss of carbon dioxide both of which actions would tend to lower the ratio. From the observed pH's there could not have been serious carbon dioxide loss, but at any rate the ratio of 0·84 found should be

slightly below the physiological. Or, had the blood been collected under anaerobic conditions, we should expect the mean distribution of bromide between cells and plasma to be close to 0·85.

Probable error from bromide shift

Our immediate purpose in determining the distribution ratio of bromide between corpuscles and plasma was to assess the extent of the error that might arise from the bromide shift. As the bromide ratio is much nearer unity than the chloride ratio, the bromide shift is likely to be the source of a smaller error in serum bromide determination than is the chloride shift in serum chloride determination. Also, the pH change in venous blood with loss of a little carbon dioxide would not be expected to be large, as it tends to be compensated by the concurrent formation of more oxyhaemoglobin. If we take an instance when $(Br)_c/(Br)_p = 0{\cdot}84$, the packed cell volume is 45%, the cells contain 70% and the plasma 93% of water, and the cell water and plasma water contain 16·8 and 20 mg. Br/100 ml., respectively, it can readily be calculated that, if the bromide becomes equally distributed between cell and plasma water, the fall in level of plasma bromide would be 1·1 mg. Br or 5·9%, and a physiological $Br_p/Br_{c.s.f.}$ of 2·50 would be decreased to 2·35. In bloods where the $(Br)_c/(Br)_p$ is appreciably less than 0·84, especially when the packed cell volume is high, the error could be perhaps over 10%, but with the procedure used in the present research it seems improbable that any error arising from bromide shift is likely to exceed 2–3%. This estimate of possible error from bromide shifts may be compared with the observed values shown in Table 4. Here the heparinized bloods were kept at room temperature to hasten equilibrium. After 4 days' storage the plasma bromide values were close to the expected equilibrium values, and only one sample had a value greater than 10% below the initial value. With clotted blood at 4°, as might be expected, the bromide shift to the corpuscles is slower. Even with prolonged storage of up to 12 days, the greatest fall was 4·2%.

We may thus conclude, on the evidence of rapid equilibration of administered bromide with corpuscles and plasma, and from the magnitude of the ratio of the distribution of bromide between corpuscles and plasma, that, under the conditions of collection and storage of the blood at 4°, no serious errors in the ratio $Br_s/Br_{c.s.f.}$ are likely to arise from bromide shift after the blood has been drawn.

Equilibration of bromide with extracellular fluid

There is evidence in the literature that bromide equilibrates within a few hours with extracellular fluid. For example, Brodie, Friedman & Ferraro (1941) have suggested its use as a measure of extracellular fluid some 3 hr. after oral administration in man. Greenberg *et al.* (1943), from experiments on dogs with ^{82}Br, found it to be completely distributed in the body within 1 or 2 hr. We have followed in a few subjects the blood level at short intervals after intravenous administration of sodium bromide, and a typical case (Table 5) shows that the serum-bromide level falls rather rapidly for about 2 hr. following intravenous administration. After about 2 hr. the rate of fall rapidly decreases. During the first 2–3 hr., presumably, equilibrium is taking place with the body's extracellular fluids.

Table 5. *Rate of fall of blood bromide following intravenous administration*

Time after injection (hr.)	Bromide (mg./100 ml.) Serum	Fall/hr.
0·25	24·8	—
1·0	22·8	2·7
2·0	20·4	2·4
8·0	19·2	0·6
24·0	18·2	0·06

Persistence in blood of administered bromide

Some 24 hr. after giving the bromide we have found the rate of fall to be commonly less than 1·0 mg. Br/100 ml. serum/day. Thus if the initial blood level is over 30 mg./100 ml. serum, determinable amounts of bromide will remain for some weeks. We shall later consider the matter of further administration of bromide in prolonged studies on patients, with the related question of a possible variation of the ratio $Br_s/Br_{c.s.f.}$ with different blood bromide levels, but the persistence of administered bromide in the body fluid is an important aspect of the barrier test, and a matter of some physiological interest. Palmer & Clarke (1933) demonstrated in the dog that the percentage of bromide in urine halides is less than the percentage of bromide in blood halides. They found, however, that the ratio of those urine and blood percentages was constant on a constant chloride intake. On a salt-poor diet it was 0·40 and on a high salt diet it was about 0·70. Similar ratios were found in a few patients with bromide dermatitis. It therefore appeared that the kidney preferentially excretes chloride. A similar conclusion is reached by Smith & Walker (1938) and by Bodansky & Modell (1941). Bromide is not removed from the plasma by increased excretion of urine but only by replacement by dietary chloride. Palmer & Clarke (1933) recorded about 120 mg. Br/100 ml. blood 30 min. after intravenous administration to a dog on a salt-poor diet, and 4·5 months later the blood still contained about 16 mg./100 ml. We have determined serum/c.s.f. ratios on patients who had about 7 mg. Br/100 ml.

serum more than 50 days after they had received bromide to the extent of about 35 mg./100 ml. of serum, and who had had the ordinary hospital salt intake. We commonly find that the relationship between serum bromide and time after bromide administration is of a linear rather than an exponential type, indicating that the rate of bromide loss is largely independent of its level in the serum.

Whether or not the kidney excretes bromide in preference to chloride has long been a vexed question. Mason (1936) supports the findings of Palmer & Clarke (1933) but Frey (1932) and Moller (1932) believe that the kidney does not distinguish between chloride and bromide. The question should, perhaps, be re-examined with modern methods for bromide determination.

Gastric secretion of bromide

Since the observations of Nencki & Schoumov-Simanowsky (1894) on the presence of bromide in the gastric juice of dogs given bromide, there has been repeated mention of gastric secretion as a possible factor in lowering serum bromide. Davenport & Fisher (1940) have clearly shown that in Pavlov pouches in dogs the rate of secretion of bromide is of the same order as the rate of secretion of chloride, and the concentration of bromide in the gastric juice is of the order of 50% higher than that in plasma. The recent observation of Gabrieli (1950) that this bromide is not secreted with the hydrochloric acid of the juice does not affect this point in our argument, but is suggestive in the study of the physiology of bromide. It seems unlikely that the volume of gastric juice in any short period could be more than 3–4% of the volume of the extracellular fluid and only the 50% excess over the plasma level could be regarded as likely to change the plasma level. Thus we should not expect a fall in plasma bromide level of more than 1–2% following a very active period of gastric secretion and such an alteration is within the usual analytical variation. Fleischacker & Scheiderer (1928) maintain that blood for the bromide barrier test should be taken in the post-absorptive state, but their values (by the auric chloride method) of less than 5% drop following food can hardly be regarded as significant.

Equilibrium between plasma and cerebrospinal fluid

Walter (1929) recommended an interval of 24 hr. between the last oral dose of bromide and lumbar puncture. This interval seems to have been generally accepted, though there is a paucity of evidence as to the length of time really necessary. Stern & Gautier (1921) demonstrated the presence of bromide in the c.s.f. of animals 1·25, 3 and 16 hr. after intravenous administration of relatively large doses. Wallace & Brodie (1940), after intravenous injection of 22 g. of sodium bromide to a dog weighing 22 kg., found $Br_s/Br_{c.s.f.}$ to be nearly constant after 7 hr. and constant from 24 hr. to 13 days. The c.s.f. was drawn from the *cisterna magna*. With ^{82}Br, Greenberg *et al.* (1943) found a plateau level in about 2 hr. in the dog. Table 6 shows some of our results with human subjects. It is

Table 6. *Equilibrium with cerebrospinal fluid after intravenous injection of sodium bromide*

Values in parentheses are for ventricular fluid.

No.	Diagnosis	$\frac{Br_s}{Br_{c.s.f.}}$	Time after injection of NaBr (days hr.)
56S	Head injury	$\frac{24·3}{5·1}=4·80$	5
		$\frac{23·1}{7·7}=3·00$	28
59S	Subacute combined degeneration of spinal cord	$\frac{22·8}{5·6}=4·07$	5
		$\frac{22·0}{9·6}=2·29$	24
79X	Tuberculous meningitis	$\frac{24·0}{16·9}=1·42$	12
		$\frac{22·6}{15·6}=1·45$	28
34X	Pneumococcal meningitis	$\frac{19·7}{16·0}=1·23$	20
		$\frac{19·0}{15·0}=1·27$	44
90X	Schizophrenia	$\frac{23·8}{7·5}=3·17$	21
		$\frac{22·4}{8·0}=2·80$	48
28X	Pulmonary tuberculosis	$\frac{24·0}{8·7}=2·76$	25
		$\frac{21·4}{8·0}=2·68$	48
105X	Lymphocytic meningitis	$\frac{18·2}{8·0}=2·28$	24
		$\frac{11·1}{5·1}=2·18$	10
98X	Tuberculous meningitis with hydrocephalus	$\frac{27·7}{13·8}=2·01$ (11·10)	5
		$\frac{25·7}{17·7}=1·45$ (3·72)	24
		$\frac{24·5}{18·1}=1·35$ (2·85)	48
		$\frac{11·1}{8·3}=1·34$ (2·31)	7

clear that an interval of about 5 hr., at which we have made many other observations with similar results, is insufficient. In the meningitis cases, 79X, 34X, equilibrium was attained in 12 and 20 hr., respectively, but in this condition, as will be shown elsewhere, the barrier permeability is greatly increased and such cases may give a false impression of the time interval necessary with the barrier normal. In the case of schizophrenia, 90X, 21 hr. was insufficient, and in cases 28X and 105X, 25 and 24 hr. were barely sufficient. In the case of hydrocephalus, 98X, in which we got a more continuous record for both lumbar and ventricular fluids, 24 hr. was insufficient; in 48 hr. equilibrium was reached with the lumbar fluid but not with the ventricular. We have followed another case of hydrocephalus at shorter time intervals up to 24 hr. with similar results.

Although the evidence from Table 6 is not as extensive as might be desired for the more normal cases, it suggests that 24 hr. should be the minimal interval between intravenous bromide dosage and lumbar puncture for the purposes of the bromide test. With oral dosage, a considerably longer interval should be set.

Concentration of bromide in cerebrospinal fluid at different levels in the neural axis

Masserman (1934), in many observations on paretic and schizophrenic patients, found that the lumbar fluid contained slightly more bromide than the cisternal and in seven cases Bau-Prussak & Prussak (1927) reported higher bromide values in the lumbar than in the cisternal fluids. Our own observations are mostly from rather extreme neuropathological conditions which will be discussed elsewhere. However, for Table 7 we have selected six cases where the fluids, as judged from the diagnosis, condition of the patient and concentration of protein in the fluids, are likely to be sufficiently near normal for a comparison of them to be valid. It may be seen that the bromide concentration of the ventricular fluid is about half that of the lumbar, with a resultant distribution ratio between 4·0 and 5·0. In two cases the bromide level of cisternal fluid was about midway between that of lumbar and ventricular fluid. It is clear that values obtained from either ventricular or cisternal fluid should not be compared with those obtained from lumbar fluid.

Table 7. *Bromide and protein concentrations of cerebrospinal fluid at different levels in the neural axis*

Bromide and protein values are expressed as mg./100 ml.

Patient no. and diagnosis	Serum (*S*)	Cerebrospinal fluid								
		Lumbar (*L*)		Cisternal (*C*)		Ventricular (*V*)				
	Bromide	Bromide	Protein	Bromide	Protein	Bromide	Protein	*S/L*	*S/C*	*S/V*
36X. Rhinorrhoea	12·0	5·4	48	—	—	2·7	—	2·22	—	4·44
39X. Fits	28·7	12·1	22	—	—	7·1	22	2·37	—	4·04
34X. Intracranial tumour	11·7	4·5	16	—	—	2·8	11	2·60	—	4·18
65X. Psychosis	33·8	12·1	35	—	—	6·9	17	2·71	—	4·90
50X. Cystic glioma	25·6	11·0	60	8·7	60	7·8	92	2·33	2·94	3·28
88X. Tuberculous meningitis, partly healed	35·5	23·5	69	14·6	45	10·5	23	1·51	2·43	3·38

Table 8. *Effect of repeated tappings on bromide level of lumbar cerebrospinal fluid*

Patient no. and diagnosis	Sample no.	Approx. vol. tapped (ml.)	Interval between samples (min.)	Bromide (mg./100 ml.)
36X. Cerebrospinal fluid rhinorrhoea	1	10	—	6·0
	2	10	10	5·3
	3	5	20	4·7
39X. Infantile hemiplegia	1	15	—	8·9
	2	10	20	8·7
	3	10	10	8·7
	4	5	10	8·7
101S. Disseminated sclerosis	1	3	—	10·8
	2*	7	2	—
	3	4	2	9·7

* Not used for bromide determination.

Table 9. *The distribution of bromide between blood and cerebrospinal fluid in control patients*

Condition	No. of cases	$\frac{Br_s}{Br_{c.s.f.}}$ Mean	S.D.	No. of cases with ratio <2·15	2·15–2·44	2·45–2·74	2·75–3·04	>3·04
Disseminated sclerosis	20	2·60	0·30	1	5	7	7	0
Psychosis	10	2·60	0·30	0	3	3	3	1

Effect of repeated lumbar punctures on the bromide level of cerebrospinal fluid

Fleischacker & Scheiderer (1930) found a fall of from 4·5 to 9·0 % in withdrawing successively three 5 ml. samples of lumbar fluid, and Walter (1929) has a warning on this point. On two of three patients our observations (Table 8) were made in the course of lumbar air encephalograms (cases 39X, 101S). In one of these and in case 36X there was a marked fall in bromide, while in case 39X the fall was less than might have been expected with the removal of relatively large amounts of fluid. It is clear, however, that a considerable error may arise, where large amounts of fluid are removed, if the first drawn fluid is not used for the bromide analysis.

The normal distribution of bromide between serum and cerebrospinal fluid

With the precautions implied above, the ratio $Br_s/Br_{c.s.f.}$ has been determined in over 200 cases, and in a number of these many times. All the cases, however, were hospital patients and like most workers in this field we have no control values from healthy subjects. From a variety of apparently benign neurological conditions like headache, neuralgia, anxiety neurosis, epilepsy, motor neurone disease, we found rather a high standard deviation and chose as controls a less heterogeneous group consisting of ten psychotic patients with normal c.s.f. protein and twenty cases of disseminated sclerosis, in which the c.s.f. protein did not exceed 50 mg./100 ml. (Table 9). It is no doubt fortuitous that the two groups gave precisely the same ratios and standard deviations. In choosing such patients as controls it may be remarked that, according to Walter (1929), there is a tendency towards high ratios or decreased permeability in schizophrenic patients and the ratio is in the normal range in cases of multiple sclerosis provided the c.s.f. protein is normal. In using cases of psychosis and disseminated sclerosis as controls we are not suggesting that in such conditions the barrier permeability is normal. It would seem in fact that our controls were comparable with those chosen by most workers, in that they gave a mean serum/c.s.f. bromide ratio of about 3·10 by the auric chloride method (2·60 by the iodometric method).

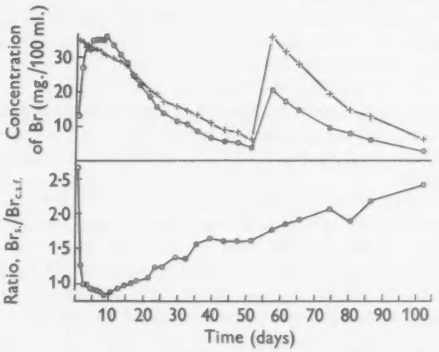

Fig. 1. Case of a psychotic patient who received orally 1·0 g. NaBr thrice daily for 3 days. About 60 hr. after the last dose bromide values in serum and c.s.f. were 35·2 and 13·3 mg. Br/100 ml., respectively (ratio 2·65). The patient was then given tuberculin intrathecally, and the bromide levels in serum and c.s.f. were followed over a period of 102 days. Between 50 and 60 days the patient was given another similar oral course of NaBr. ×—×, serum; ⊙—⊙, c.s.f.

Repeated tests on the same patient

Few repeated Walter bromide tests on the same patient have been recorded. Walter (1925) reported repeat values on four patients at times varying from 9 to 37 days after the initial observations, and concluded that the ratio was very constant and uninfluenced by time and amount of bromide present. Further observations of this kind are desirable. Fig. 1 illustrates our findings in a case where a rapid and profound change in barrier permeability occurred following intrathecal administration of tuberculin, the medical aspects of which will be discussed elsewhere. The main features of this case have been repeatedly observed in other patients. The following points may be observed:

(*a*) The consistently diminishing values obtained on the serum, except at about a week from the start when the blood values remained constant over several days and indeed on one day showed a slight rise. Such changes could well have been due to changes in the water balance as a result of the treatment. As remarked earlier the fall in blood bromide tends to be linear with time. Walter (1929) records a similar curve obtained from his own blood over about 50 days.

(*b*) The c.s.f. values are likewise on a continuous curve with only two points slightly erratic. The peak c.s.f. value of 35·9 mg. Br/100 ml. was the mean of close triplicate determinations. It is not only much higher than the corresponding serum value (30·5) but higher than the serum peak at the start. This surprising bromide distribution will be discussed elsewhere.

(*c*) The values of the ratio $Br_s/Br_{c.s.f.}$ also lie on a smooth curve, despite a large change in the blood level between the 50th and 60th day.

(*d*) When the observations were stopped, the ratio, 2·40, was still somewhat lower than its initial value, indicating that the barrier had not yet quite recovered from its treatment more than 3 months earlier.

From such considerations Fig. 1 is taken as evidence of the general reliability of the bromide test. It also proves, and we have further similar evidence on the same point, that the ratio is independent of the level of bromide in the blood—at least within the limits recommended for the test.

SUMMARY

1. The bromide test of meningeal permeability (Walter, 1929) has been critically examined.

2. The auric chloride method for estimation of bromide in serum is unreliable, and values for the serum/cerebrospinal fluid bromide ratio determined by this method are too high.

3. When bromide is added to human blood an equilibrium between plasma and erythrocytes is established in a few minutes. At this equilibrium the ratio (Br^- in cell water)/(Br^- in plasma water) is 0·84 (S.D. 0·08) and from the magnitude of this ratio it is inferred that administered bromide replaces more than its equivalent of chloride in the human erythrocyte. The ratio also varies inversely with the pH of the blood.

4. On storage of blood the serum bromide tends to fall to a level corresponding to an even distribution throughout the blood water. With clotted blood stored in a refrigerator this process is probably not complete for 1 or 2 weeks. When the serum is separated within 1–2 days the bromide level is not likely to have dropped more than 2–3 %.

5. Intravenously injected bromide takes several hours to equilibrate with the extracellular fluid of the body.

6. Administered bromide persists in the blood for several weeks. Our evidence favours the view that the kidney excretes chloride in preference to bromide.

7. Intravenously injected bromide, except where there is increased barrier permeability, normally takes about 24 hr. to equilibrate with the lumbar cerebrospinal fluid and longer with ventricular fluid.

8. The bromide concentration of cerebrospinal fluid decreases on ascending the neural axis. In nearly normal cases the bromide concentration in ventricular cerebrospinal fluid is about half that of lumbar.

9. A significant error in the bromide distribution ratio between serum and cerebrospinal fluid can arise if much lumbar fluid is removed before the specimen is taken.

10. In thirty cases with nearly normal meninges the bromide distribution ratio between serum and cerebrospinal fluid was 2·60 (S.D. 0·30). This ratio is independent of the level of bromide in the serum.

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The Source of Antibody Globulin in Rabbit Milk and Goat Colostrum

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After the intravenous injection of radioactive amino acids into lactating rabbits, there is an extremely rapid and efficient incorporation of radioactive carbon into milk proteins. The blood plasma-protein radioactivity reached its maximum value 6 or 7 hr. after the injection, but it is only about one-tenth the activity of a milk protein sample collected at the same time (Campbell & Work, 1951, 1952). It has been concluded that blood plasma protein is not a major and direct precursor of milk protein and that most of the milk protein is synthesized in the mammary gland from amino acids or small peptides. Amino acids isolated from radioactive rabbit casein were, however, of slightly higher specific activity than the corresponding amino acids from whey protein, and it seemed possible that this difference might be due to the direct transfer from blood to milk of a fraction of the plasma protein. Campbell & Work (1952) calculated that up to 25% of the total whey protein would have to be so transferred to account for the lowered radioactivity of the whey. Experiments have, therefore, been directed towards identification of the protein transferred from blood to milk in the lactating rabbit. More recently we have been interested in the biosynthesis of milk proteins in the goat, and it was hoped to extend the work to this animal.

Extensive studies on the transmission of immunity from parent to offspring have suggested that antibody may pass across the placental barrier unchanged; high concentrations of antibody being found in colostrum and traces in milk (Ehrlich, 1892; Ratner, Jackson & Gruehl, 1927; Marrack, 1947; McMeekin & Polis, 1949, McCarthy & McDougall, 1953). According to Smith (1948) the immune-globulin fraction of cow colostrum is identical with the immune-globulin of cow milk and closely similar to, but not quite identical with, the T-globulin of cow plasma. The immune-globulins are, however, proteins of high molecular weight, rabbit γ-globulin having a molecular weight of 160000 (Kabat, 1939; Nichol & Deutsch, 1948) and there has been considerable doubt as to whether this whole protein passed across cell barriers or whether it was partially degraded and resynthesized (Cohen, 1950; Calman & Murray, 1951; Brambell, Hemmings & Henderson, 1951).

From the work on immune-globulin, it was clear that in so far as proteins could be transferred directly from rabbit blood to milk, they would probably be found in the immune-globulin fraction. Moreover, any protein transferred directly to the milk during the hours immediately following intravenous injection of a radioactive amino acid should have the radioactivity characteristic of the plasma proteins and, 6 hr. after injection, should have not more than about one-tenth the activity of protein synthesized in the mammary gland. Accordingly, a lactating rabbit was immunized with pneumococcus (type III) and was then given an injection of [^{35}S]methionine. Blood and milk samples were withdrawn at intervals and pneumococcus capsular polysaccharide (SSS III) was used to precipitate the specific antibody from blood and milk. The antibodies thus obtained were compared with one another and with the other milk and blood proteins. A preliminary account of this investigation has been published (Campbell, Humphrey & Work, 1953).

An attempt was then made to reproduce in a goat the experimental conditions already used with rabbits. However, even after a prolonged course of intravenous injections of formalin-treated pneumococcus (type III) the serum antibody level did not rise above 0·4 mg./ml. The level in the milk was much lower, and was too low to permit accurate

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quantitative work. We therefore used an alternative approach and measured the distribution of radioactivity in goat colostrum proteins after injection of radioactive methionine.

Colostrum differs from milk not only in its higher protein content but also in the relative proportions of the constituent proteins (Crowther & Raistrick, 1916). Smith (1946) calculated from electrophoretic data that 55% of the protein in cow colostrum taken 1 hr. *post partum* consists of an immune-globulin fraction rich in antibodies, whereas normal milk contains only about 10% of immune-globulin. The goat has been less extensively studied, but Deutsch (1947) showed that there was a similar and rapid change in the composition of proteins secreted by the mammary gland immediately *post partum*.

In the present experiments a goat was injected with [^{35}S]methionine shortly after parturition and the colostrum samples taken at 10 and 13 hr. after the injection were separated into casein, lacto-globulin and immune-globulin fractions. The distribution of radioactivity in these proteins was consistent with the assumption that in the goat as in the rabbit immune-globulin passes from blood to milk without degradation and resynthesis.

EXPERIMENTAL

A lactating rabbit with a litter 1 week old was injected intravenously with a suspension of formalin-treated pneumococcus type III. Eight injections were given during a period of 17 days, the total number of cells injected being 8×10^{10}. The antibody content of the serum was measured by the method outlined below and was 4·5 mg./ml. after 2 weeks, rising to 10 mg./ml. at 3 weeks. Antibody concentrations are expressed as mg. dry weight of antigen-antibody complex containing about 4% of antigen (Fig. 1).

The rabbit was then given, by intravenous injection, 6·7 mg. of [^{35}S]DL-methionine containing about 25 μc of ^{35}S (supplied by the Radiochemical Centre, Amersham) on the fourth day after the last injection of antigen. The animal was separated from its litter and milked after 6 hr. Immediately after milking, a blood sample was taken from the ear. Milking and bleeding were repeated at 30 hr. and 9 days.

The animal was kept in full lactation throughout the experimental period by replacement of the litter every 14 days by another litter 7 days old.

Treatment of rabbit milk. After removal of the bulk of the fat by low-speed centrifugation, most of the casein was separated by centrifugation at 1° for two periods of 1 hr. in an angle head at 33000 *g*. The clear supernatant fluid was treated with 'Merthiolate' (Eli Lilley and Co. Ltd., Basingstoke) 1:10000; and stored at 2°. It showed no tendency to spontaneous precipitation. Antibody was precipitated by addition of SSS III at optimum proportions (Dean & Webb, 1926), and after allowing 24 hr. at 2° for precipitation, the antigen-antibody complex was centrifuged down, washed twice with 0·9% NaCl at 2°, and once with water. It was dried with ethanol and ether and weighed. The antigen-antibody complex was calculated to contain approximately 4% of antigen. The milk contained about one-twentieth of the concentration of antibody in the plasma, and the yields varied between 8·7 mg. from 17 ml. milk at the height of immunization and 3·0 mg. from 14 ml. milk during the declining phase.

The whey protein remaining in solution after removal of antibody was precipitated by heat.

Treatment of rabbit blood. The serum was separated and antibody to SSS III was precipitated from a 3·5 ml. sample by addition of SSS III at optimum proportions and collected in the same way as for milk.

The supernatants after precipitation of the antibody were treated with 2 vol. of 27% (w/v) Na_2SO_4 dissolved in Na phosphate buffer, pH 7·8 ($I = 0·1$), to produce a final concentration of 18% (w/v). The precipitated globulins, which consisted of about 95% of γ- and 5% β-globulins, were redissolved and reprecipitated at 18% (w/v) Na_2SO_4. After dialysis against distilled water this 'globulin' fraction was coagulated with trichloroacetic acid, washed and dried. The proteins remaining in solution at 18% Na_2SO_4 were coagulated by heat, washed and dried. They are termed the 'albumin' fraction, although some α- and β-globulins are included with them.

Specificity of precipitation. Since differences in the radioactivity of the immune-globulin and the other plasma proteins were to be measured it seemed desirable to arrange a control experiment to indicate to what extent proteins might be bound to an antigen-antibody precipitate by non-specific adsorption. This was achieved by adding ovalbumin to the milk whey and blood serum and precipitating it by the addition of antiovalbumin antibody. The radioactivity of this precipitate then gave a measure of the degree of non-specific adsorption of radioactive protein. The experiment was conducted as follows.

A sample of whey (17 ml.) diluted with an equal volume of 0·9% NaCl was mixed with 30 mg. (dry wt.) of rabbit γ-globulin, of which one-third was antiovalbumin antibody,

Table 1. *Precipitation of pneumococcus antibody from rabbit serum and milk by specific soluble polysaccharide type III. Effect of addition of antiovalbumin followed by ovalbumin on yield and radioactivity of pneumococcal antibody*

(For conditions see text.)

		Direct precipitation with SSS III		After treatment antiovalbumin-ovalbumin	
Material	Time (hr.)	Yield (mg./ml.)	Radioactivity (counts 1 min./0·3 sq.cm. of infinite thickness)	Yield (mg./ml.)	Radioactivity (counts 1 min./0·3 sq.cm. of infinite thickness)
Milk	6	0·505	10	0·49	7
	30	0·535	20	0·525	17
Serum	6	10·7	19	10·4	16
	30	9·9	22	9·4	17

and treated with 1 mg. crystallized ovalbumin dissolved in 0·9% NaCl (0·5 ml.). The precipitate, which was not significantly radioactive, was removed after 24 hr. at 2° and the pneumococcus antibody then precipitated as before. This procedure diminished the yield of anti-SSS III by about 5% and lowered the radioactivity about 20%, compared with the product obtained from the untreated sample.

Duplicate samples of blood serum (3·8 ml.) were also treated with the antiovalbumin-ovalbumin system in the same way. The effect upon the yield and radioactivity of the anti-SSS III precipitates was very similar to that on the milk samples (Table 1).

It is difficult to know what importance to attach to the diminution of radioactivity in anti-SSS III precipitates from the pretreated milk and serum samples. Such a diminution might be expected if the pretreatment process prevented non-specific co-precipitation of traces of some more highly radioactive material, e.g. casein in the milk—but in blood serum the main protein fractions would all have radioactivities of the same order and this explanation is therefore unlikely. Since the counts and consequently their accuracy, were relatively low, and since the findings do not affect our main conclusions, we did not pursue the matter further.

Goat colostrum

A British Saanen goat gave birth to three kids over a period of approximately 1 hr. About 2 hr. after the birth of the first kid the mammary gland was milked dry by an experienced milker and about 1 l. of very thick colostrum obtained. The goat was then given intravenously 17 mg. of DL-methionine containing approximately 40 μc of ^{35}S. The kids were allowed to remain with the mother, and 10 hr. after the injection 35 ml. of colostrum were obtained: 13 hr. after the injection 5 i.u. of posterior pituitary extract were given intravenously, a blood sample was taken and 290 ml. of colostrum were withdrawn.

Treatment of goat colostrum. The colostrum was fractionated according to Smith (1946). After removal of the fat by centrifugation, the casein was precipitated at pH 4·5. The casein was washed twice with distilled water, the first washing being added to the whey fraction. After the casein had been dissolved in water by the addition of N-NaOH to pH 6·5, the solution was clarified by filtration through kieselguhr and again precipitated. The twice-washed casein was freeze-dried.

The whey was clarified by filtration through a pad of paper pulp, adjusted to pH 6·0 with 0·5 N-NaOH and solid $(NH_4)_2SO_4$ was added to 0·49 sat. (30·4 g./100 ml.). The precipitate, which will be referred to as the immune-globulin fraction, was separated by centrifugation. The supernatant was filtered until completely clear and then saturated with $(NH_4)_2SO_4$. The precipitate will be referred to as the lactoglobulin fraction. The immune-globulin and lactoglobulin precipitates were dissolved in water, clarified by filtration and again precipitated at the appropriate concentration of $(NH_4)_2SO_4$. After dissolving the precipitates in water the solutions were extensively dialysed at 1° and then freeze-dried.

Treatment of goat blood. The blood sample was fractionated into plasma protein and red cells as previously described (Campbell & Work, 1952).

Methionine and cystine in goat colostrum proteins. Comparison of the radioactivity of whole proteins could be misleading if these differed greatly in amino-acid composition. To check that methionine-labelled immune-globulin could be compared with methionine-labelled lactoglobulin, a rough estimate was made of cystine and of total sulphur in the two proteins. A sample (15 mg.) of each protein was digested with Pirie's (1932) reagent followed by HCl as described by Simpson, Tarver & Rutman (1950) and sulphur estimated as benzidine sulphate (Scott & Furman, 1939). Immune-globulin was found to contain about 0·7% sulphur by weight and lactoglobulin about 1·3%. Another sample of each protein (10 mg.) was hydrolysed for 24 hr. at 110° with 6 N-HCl. Excess of acid was removed by evaporation *in vacuo* and the amino acid mixture treated with excess of bromine water to oxidize cystine and cysteine to cysteic acid (Friedmann, 1903). A comparison between the amount of cysteic acid in the two hydrolysates was then made by paper chromatography followed by reaction with ninhydrin and visual comparison of the cysteic acid spots. This is a very roughly quantitative method but as the differences in radioactivity were sixfold or more it was sufficiently accurate for our purpose. No significant difference was found in the cysteic acid concentration in the two protein hydrolysates.

Assay of radioactivity

All protein samples were transferred to polythene disks, rabbit proteins to 0·3 sq.cm. disks, goat proteins to 1 sq.cm. disks, and counted at infinite thickness, using a standard end-window counter. Since all the proteins were dried in a similar manner, no special precautions were taken to control moisture content during counting.

RESULTS

Experiments with the rabbit. Fig. 1 shows the variation in concentration of rabbit-serum antibody over the period of the experiment. It will be seen that the injection of radioactive methionine took place when antibody concentration was at its maximum, and that during the following 9 days of milking the antibody level in the serum was falling rather rapidly.

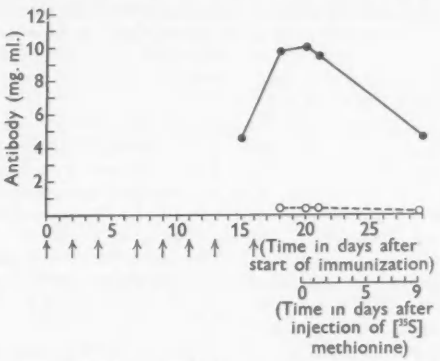

Fig. 1. Antibody content of serum and milk of rabbit immunized with pneumococcus. ●—●, blood serum antibody; ○---○ milk antibody. The times of the intravenous injections of preumococci are indicated by arrows on the time axis.

Table 2. *Activity of milk and blood protein after administration of* [^{35}S]*methionine to a lactating immune rabbit*

Results as counts/min./0·3 sq.cm. of infinite thickness.

Material	Time after injection		
	6 hr.	30 hr.	9 days
Serum antibody	16	17	15
Milk antibody	7	17	14
Casein	108	30	6
Whey protein (after removal of antibody)	91	38	8
Serum, globulin	17	16	10
Serum, albumin	18	13	7

Table 3. *Distribution of proteins in goat colostrum*

Time after parturition (hr.)	Total amount of protein (g.)	% of each component		
		Casein	Immune-globulin	Lactoglobulin
1	105	24	62	14
11	2·15	59	25	16
14	13·10	52	26	22

Table 4. *Radioactivity of blood and colostrum proteins obtained from a lactating goat after injection of* [^{35}S]DL *methionine*

Results as counts/min./sq.cm. of infinite thickness.

Fraction	Time after injection	
	10 hr.	13 hr.
Casein	58	48
Immune-globulin	6	4
Lactoglobulin	37	40
Plasma protein	—	14
Red cells	—	1

In Table 2 a comparison is made of the radioactivity of the milk and serum proteins of the rabbit. It is clear that milk-antibody radioactivity follows closely the serum-antibody activity and fails to show the steep rise in activity characteristic of casein and of whey protein.

Experiments with the goat. The amount of protein isolated from each of the colostrum fractions is shown in Table 3, while in Table 4 a comparison is made of the radioactivities of the various proteins isolated. The casein and lactoglobulin fractions are notably more radioactive than the immune-globulin.

It seemed just possible that this difference might be due to a difference in the methionine content of the proteins but this is not so. To account for the higher activity of lactoglobulin and casein we should have to assume that these proteins contained between 6 and 10 times as much methionine as immune-globulin but roughly quantitative measurement of cystine and of total sulphur in lactoglobulin and immune-globulin showed no difference of this order between the two proteins (see Experimental).

DISCUSSION

We have previously shown that, 6 hr. after the injection of a radioactive amino acid to a lactating rabbit, the protein synthesized in the mammary gland had up to 10 times the radioactivity of the blood protein. In the present experiments (Table 2) 6 hr. after injection of [^{35}S]methionine the casein was 15 times more active than the milk antibody and the whey protein not precipitable by pneumococcus antigen was 13 times as active as the milk antibody.

These results are so striking as to leave no doubt that, during normal lactation, rabbit pneumococcus antibody passes through the mammary gland without degradation and resynthesis. The results obtained during the remainder of the experimental period served to confirm this deduction. Both the 30-hr. and the 9-day samples of milk antibody were of the same radioactivity as the serum antibody, moreover, at 9 days the milk antibody was twice as active as the casein or the residual whey protein, and also twice as active as the serum albumin and more active than the serum globulin (Table 2).

The high radioactivity of antibody at 9 days is probably explained by the shape of the curve for total antibody (Fig. 1). It is known that although specific antibody is synthesized independently of other plasma globulins, all the γ-globulins, including antibody globulin, are catabolized at the same rate, equal to 10–12½% per day (Humphrey & MacFarlane, 1952). Synthesis of new antibody slowed down rapidly during the experimental period so that antibody globulin labelled with ^{35}S on the first day

was diluted at rapidly diminishing speed with new antibody globulin, whereas other labelled globulins were diluted at a steady rate governed by the constant production of new plasma protein. It is not surprising that the 6-hr. sample of milk antibody was less radioactive than the 6-hr. sample of serum antibody. The milk collected at 6 hr. had accumulated in the mammary gland over, at least, a 6-hr. period so that it would not be in equilibrium with serum collected at 6 hr. but would represent an average value for the 6 hr. period. During part of this time unlabelled or weakly labelled antibody protein must have been passing into the gland.

The results with goat colostrum are not as clear cut as those with rabbit milk. This was not unexpected since the proportions of different proteins in colostrum varies very considerably with time (Table 3). Thus, at 1 hr. the immune globulin accounted for 62% of the milk protein, whereas at 11 and 14 hr. it was only 25% of the total protein. In spite of this difficulty the results are consistent with those obtained in the rabbit and suggest that in the goat, as in the rabbit, immune-globulin passes unchanged through the mammary gland. How this can occur without (in colostrum at any rate) a corresponding accumulation of other plasma proteins raises another problem upon which our results, shed no light.

Smith and his colleagues have shown that immune-globulin of bovine colostrum is very similar to the T-globulin of blood plasma, but they find differences between the two in amino acid composition and in absorption spectrum (Smith, Greene & Bartner, 1946; Smith & Coy, 1946; Smith, 1948). They suggest that the mammary gland alters the protein as it passes through the gland but, in the present work, if any such alteration does take place it does not involve rupture and reformation of methionine peptide linkages.

In view of the demonstration that plasma protein can pass into the milk without degradation and resynthesis, the results quoted by Campbell & Work (1952) for unequal specific radioactivities of amino acids isolated from rabbit casein and rabbit-whey protein after injection of radioactive amino acids, could be supposed to be due to such transfer. It has thus become necessary to repeat these experiments with a larger animal and to fractionate the milk-whey proteins before determination of radioactivities. The results of such an experiment will be reported later.

SUMMARY

1. The antibody concentration in the milk of an immune rabbit (type III pneumococcus) was about one-twentieth of that in the blood.

2. After the injection of radioactive [^{35}S]-methionine the distribution of radioactivity in blood and milk proteins was measured.

3. Antibody was selectively precipitated from blood serum and from milk whey by addition of type III specific soluble polysaccharide.

4. Serum antibody and milk antibody acquired a similar degree of radioactivity. Casein and lactoglobulin collected 6 hr. after injection of methionine had 6–10 times the radioactivity of milk or serum antibody collected at the same time.

5. The results show that, in the rabbit, antibody protein passes from blood to milk without degradation and resynthesis.

6. Antibody protein also passes from blood to colostrum in the goat without degradation and resynthesis.

We are grateful to Dr P. G. H. Gell for a gift of pneumococcus type III polysaccharide. It is a pleasure for us to acknowledge the technical assistance of Miss M. T. Smith, Mr J. Coote and Mr B. Price at various stages during the course of this work.

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Phosphorylation Coupled with Anaerobic Breakdown of Citrulline

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It has recently been demonstrated that preparations obtained from various micro-organisms are capable of converting citrulline into ornithine, ammonia and carbon dioxide. This reaction has been found in *Streptococcus faecalis* treated with toluene (Akamatsu & Sekine, 1951), or acetone (Oginsky & Gehrig, 1952), in freeze-dried cells of *Clostridium perfringens* (Schmidt, Logan & Tytell, 1952) and cell-free extracts of *Streptococcus lactis* (Korzenovsky & Werkman, 1952). With regard to the mechanism of the reaction, it is relevant that washed suspensions of *Strep. faecalis*, treated with cetyltrimethylammonium bromide do not attack citrulline until small amounts of adenosine diphosphate (ADP) or a larger quantity of adenosine triphosphate (ATP) is added (Knivett, 1952*a*, *b*); that cell-free extracts require inorganic phosphate and either muscle adenylic acid (AMP), ADP or ATP (Slade, 1953); and that citrulline breakdown by cell-free extracts is accompanied by the disappearance of inorganic phosphate together with the phosphorylation of ADP to ATP (Knivett, 1952*c*, 1953). Preliminary investigations in Cambridge (Knivett, 1952*c*) were concerned with the detection and isolation of ATP accompanying the breakdown of citrulline; whilst the present paper is concerned with a more detailed study of the phosphorylation of ADP to ATP. A procedure employing ^{32}P has been used which has the advantage of being independent of the action of dephosphorylating enzymes, and of making it possible to distinguish between ATP formed by the action of adenylate kinase (myokinase; see Colowick, 1951) and synthesis from ADP and inorganic phosphate.

METHODS

Preparation of the cell-free extract. Strep. faecalis (ST), National Collection of Type Cultures no. 6782, was grown in a medium containing 1·5% (w/v) L-arginine HCl, 1·0% (w/v) yeast autolysate (Marmite), 0·5% (w/v) tryptic digest of casein, 0·1% (w/v) K_2HPO_4, 0·05% (w/v) $MgSO_4$, $7H_2O$, 0·01% (w/v) $MnSO_4$, $4H_2O$, and 1·0% (w/v) glucose (sterilized separately). Satisfactory yields (5 mg. dry wt./ml.) of cells were obtained after incubation for 16 hr. at 30°.

The cells from 200 ml. of medium were harvested on the Sorvall high-speed angle head centrifuge (Ivan Sorvall Inc., New York) at 20000 *g* for 10 min. They were washed twice by resuspension in the same volume of distilled water and re-centrifuged.

The cells were disintegrated by shaking with glass beads (Ballotini no. 12, Chance Bros., Birmingham) at 50 cyc./sec. in the Mickle Sonic Disintegrator (Mickle, 1948). The cups containing 10 ml. of Ballotini beads and 10 ml. of a washed suspension of cells (approx. 20 mg. dry wt./ml.), were cooled at 0° and shaken for 15 min. The debris was filtered through a sintered glass funnel and the extract centrifuged for 20 min. at 20000 *g* to remove unbroken cells and cell-wall debris. The clear supernatant was stored at −14° until required. The protein content of the extract was measured by the method of Kalckar (1947).

Adenosine diphosphate (*ADP*) *and triphosphate* (*ATP*). The barium salt of ATP was prepared from rabbit muscle according to the directions of LePage (1949). Barium ADP was supplied by Schwarz Laboratories Inc., 202 East 44th Street, New York 17. The barium compounds were dissolved in dilute HCl and barium was precipitated by the addition of the calculated quantity of 0·5 M-Na_2SO_4. After removal of the precipitate by centrifugation, the solutions were adjusted to pH 5·8 with NaOH. About 20% of the ADP and ATP was lost by adsorption on the precipitate (Bailey, 1949). The ultraviolet technique of Markham & Smith (1949) did not reveal any AMP or ATP in the chromatogram of the ADP preparation. The ATP preparation contained about 15% ADP, but contained no detectable quantities of AMP.

$KH_2{}^{32}PO_4$. Carrier-free $KH_2{}^{32}PO_4$ was obtained from the Atomic Energy Research Establishment at Harwell. The sample had an activity of about 1 mc and was diluted with stock saline to 10 ml. The radioactivity of the incubation mixture was about 2–3 μc/ml. solution.

Incubation mixtures. The incubation mixtures contained 0·3 ml. enzyme solution (35·2 mg. protein/ml.), 0·8 ml. 0·5 M potassium succinate buffer (pH 5·8), 0·1 ml. 0·12 M-$MgCl_2$, 0·2 ml. 0·09 M-Na_2HPO_4, 0·6 ml. 0·3 M L-citrulline, 0·1 ml. $KH_2{}^{32}PO_4$ (activity about 0·1 mc/ml.), and 0·9 ml. 0·016 M-ADP or ATP. The CO_2 produced from citrulline was measured manometrically in a parallel sample in which $KH_2{}^{32}PO_4$ was omitted. Non-isotopic phosphate was also used for a control containing ATP but no citrulline.

The incubations were performed in air at 25°. ADP or ATP were added last. This initiated the reaction. Samples (0·5 ml.) were withdrawn at intervals, mixed with 30% (w/v) trichloroacetic acid (0·05 ml.) and stored at −14° until they were analysed. An initial sample withdrawn immediately after the addition of ADP and ATP.

Chromatography. Adenosine phosphates and inorganic phosphate were separated by the paper chromatographic methods of Hanes & Isherwood (1949) as modified by

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Eggleston & Hems (1952) and Krebs & Hems (1953). The second solvent consisted of 100 ml. *iso*butyric acid, 60 ml. N-NH_4OH, and 1·6 ml. 0·1 M ethylenediaminetetraacetic acid. The solvent was allowed to descend for 16 hr. at room temperature. The phosphate determinations and radioactivity measurements were carried out in duplicate according to the methods of Eggleston & Hems (1952). Suitable volumes (10 μl.) were applied to the paper with the Agla micrometer syringe (Burroughs Wellcome and Co.) in quadruplicate. A separate control mixture of adenosine and inosine mono-, di-, and tri-phosphates was applied to assist identification of the phosphate fractions.

Interpretation of specific activity data. For the purpose of the following considerations the three adenosine phosphates are represented by the symbols AP, APP and APPP, and phosphate by the symbol P. Isotopic phosphorus is indicated by an asterisk. The ratio specific activity of adenosine phosphate/specific activity of phosphate is referred to as 'relative specific activity', of the adenosine phosphate, the specific activity being expressed in counts/min./mμ. mole.

If APP is phosphorylated by P* according to the reaction

$$APP + P^* \rightarrow APPP^*, \quad (1)$$

and if no APPP is present initially the APPP* formed has the same specific activity as P*; the relative specific activity of APPP* is 1. If adenylate kinase is present APPP can also be formed without the participation of P:

$$2\ APP \rightleftharpoons APPP + AP. \quad (2)$$

The APPP formed by this reaction dilutes the APPP* formed by reaction (1) so that its relative specific activity is less than 1. When reaction (2) proceeds from right to left and if APPP* is the reactant APP* is formed. Radioactivity in APP therefore suggests the presence of adenylate kinase in the enzyme preparation, although the possibility that AP is phosphorylated directly by P* cannot be excluded. Phosphorylation of APP* by reaction (1) would yield APP*P*, and in this case the relative specific activity of APPP would be greater than 1.

RESULTS

The following substrate combinations were incubated: (1) citrulline, (2) ADP, (3) citrulline and ADP and a parallel series in which ATP (contaminated with about 15% ADP) replaced ADP. The evolution of carbon dioxide and the changes in the quantities and radioactivities of the various phosphate fractions were measured. Representative results are shown in Table 1. Citrulline alone was not changed in 60 min. At the same time a mixture containing ADP and citrulline released carbon dioxide, formed ATP and removed phosphate and ADP, the four changes being of the same order of magnitude though not exactly equivalent. For example, at 11·5 min. the changes are as follows: carbon dioxide, +1·11; phosphate, −1·85; ADP, −1·14; ATP, +1·29 μmoles/ml. Some AMP was formed after 6 min. but partly disappeared later whilst the concentration of ATP increased. After 11·5 min. there was relatively little change in the concentrations of the various phosphate fractions, although carbon dioxide formation continued. A steady state with regard to the concentrations of adenosine phosphates had been reached where the phosphorylating enzymes synthesizing ATP compensated the reaction of the dephosphorylating enzymes. This follows from the radioactivity data. The radioactivity of inorganic phosphate continually fell whilst that of ATP rose. The incorporation of isotopic phosphate into ATP steadily continued in spite of a decreased rate of accumulation of ATP.

The presence of adenylate kinase and the absence of adenosine diphosphatase in the enzyme preparation is shown by the controls from which citrulline had been omitted. When ADP alone was incubated, its disappearance was accompanied by the appearance of AMP and ATP, whilst the concentration and specific activity of inorganic phosphate remained unchanged. The labile phosphate fraction contained only traces of ^{32}P. Incubation of ATP alone caused an appearance of AMP and ADP and inorganic phosphate. This can be accounted for by the combined effects of adenosine triphosphatase and adenylate kinase. The specific activity of ADP increased steadily during incubation and was always much less than that of ATP. This observation suggests that the radioactivity was due to reaction (2) proceeding from right to left under the influence of adenylate kinase, and it is unlikely that AMP was phosphorylated directly by inorganic phosphate. In the earlier stages of incubation the relative specific activity of ATP was about 0·5. This suggests that half of the ATP was formed by the phosphorylation of ADP by isotopic phosphate and the remainder by the action of adenylate kinase (reaction 2). This would account for the formation of AMP, in the absence of adenosine diphosphatase. After 90 min. incubation, the relative specific activity of ATP had not exceeded two-thirds of the theoretical value of 2 (in the presence of adenylate kinase). This observation suggests that either adenylate kinase was rapidly destroyed or that small amounts were present. Eggleston & Hems (1952) give a value of 0·444 for the equilibrium constant, K, of the adenylate kinase system at 25°. After 75 min. incubation of the ATP preparation, a value of 0·32 for K was obtained, which suggested that an equilibrium state had not been reached. The presence of adenylate kinase has been reported in yeast (Kotel'nikova, 1949) but has not previously been reported in bacteria.

In the samples to which both citrulline and an ATP preparation containing about 15% ADP were added, the rate of carbon dioxide formation from citrulline was slower than when ADP was used, and no major changes in the amounts of adenosine phosphates occurred. No AMP was detected in these samples, in contrast to those containing ADP and citrulline or to those controls containing ATP

Table 1. *Changes in the phosphate fractions on incubation of cell-free extracts of* Streptococcus faecalis *with citrulline and adenosine di- and tri-phosphates*

For full details of conditions see text. The sample of ATP contained about 15% ADP. CO_2 measured manometrically (not corrected for retention). Relative specific activity of ADP and ATP is the ratio: specific activity of ADP or ATP/specific activity of phosphate.

Substrate added	ADP + Citrulline				ADP		ATP + Citrulline					ATP		Citrulline
Period of incubation (min.)	1	6	11·5	88	11	90	1	6·5	16	35	76	1	75	60
Quantities of phosphates (μmoles/ml. incubated solution):														
Inorganic phosphate	6·43	5·36	4·58	4·26	6·25	6·11	7·00	—	7·14	—	6·78	7·42	9·05	—
AMP	0·0	1·10	0·28	0·50	1·28	1·53	0·0	0·0	0·0	0·0	0·0	0·0	0·25	0·0
ADP	2·48	1·76	1·34	1·12	1·92*	1·12	0·37	—	0·46	—	0·67	0·78	1·05	0·0
ATP	0·31	0·97	1·60	2·39	0·55	0·77	2·67	—	2·87	—	2·79	2·58	1·41	0·0
Radioactivity (% of total):														
Inorganic phosphate	97·9	92·3	82·6	53·7	99·8	99·3	98·5	94·7	88·0	82·8	75·9	No isotope added		100
ADP	0·1	0·8	1·6	7·5	0·1	0·1	0·05	0·4	0·6	1·0	2·0	—	—	0
ATP	2·0	6·9	14·7	37·0	0·1	0·4	1·5	4·9	10·6	12·7	20·8	—	—	0
Other phosphates	0·0	0·0	1·1	1·8	0·0	0·2	0·0	0·0	0·8	3·5	1·3	—	—	0
Specific activity (counts/min./μm. mole):														
Inorganic phosphate	80	91	103	70	87	90	80	—	67	—	64	—	—	—
ADP	0·4	2·4	7·1	38·0	—	0·7	0·9	—	7·1	—	15·7	—	—	—
ATP	33·6	37·9	54·5	87·1	0·9	2·7	3·2	—	20·1	—	40·0	—	—	—
Relative specific activity:														
ADP	0·005	0·026	0·070	0·540	—	0·008	0·011	—	0·106	—	0·262	—	—	—
ATP	0·42	0·42	0·53	1·24	0·01	0·03	0·04	—	0·30	—	0·67	—	—	—
CO_2 evolved (μmoles/ml. incubated solution)	—	0·6	1·1	4·06	0·0	0·0	—	0·4	1·1	2·0	3·6	0·0	0·0	0·0

* Sample lost, estimated by difference.

but no citrulline. The amount of AMP present was calculated, assuming a value of 0·444 for the equilibrium constant, K, of adenylate kinase (Eggleston & Hems, 1952). The calculated amount of AMP present after 16 and 76 min. incubation was 0·03 and 0·07 μmole/ml., respectively.

Although there were no major net changes, ^{32}P appeared in ATP and ADP. After 75 min. incubation, 20% of the radioactivity was present in ATP and 2% in ADP. The rate of incorporation was slower than in the corresponding samples to which ADP was added, one-third of the maximal incorporation being attained after 75 min. The presence of adenosine triphosphatase was confirmed by the fall in specific activity of the phosphate.

The radioactivity of AMP was never significantly greater than the background radiation. In the later stages of incubation, an unidentified radioactive substance appeared on the chromatogram travelling in a position between inosine di- and tri-phosphate. The ultraviolet absorption of this substance was indistinct, and when sprayed with molybdate reagent gave a darker shade of blue than the other phosphates. The phosphorus content of the spot was too small to be measured accurately, so that the substance must have had a high specific activity. It may have been either glucose 1-phosphate or glucose 6-phosphate formed by the interaction of traces of glucose in the enzyme preparation and radioactive ATP synthesized during the reaction. Hexokinase is known to be present in the extracts.

Chromatograms of incubation mixtures which contained citrulline and radioactive phosphate, but no ADP or ATP, were examined for the presence of a phosphorylated derivative of citrulline; only phosphate was detected, and all the radioactivity was located in this spot.

DISCUSSION

The results show that the breakdown of citrulline to ornithine is accompanied by the phosphorylation of ADP to ATP in approximately stoicheiometric proportion. The overall change in the decomposition of citrulline may be represented by the scheme

$$\text{citrulline} + \text{ADP} + \text{phosphate} \rightarrow \text{ornithine} + CO_2 + NH_3 + \text{ATP}.$$

Experimentally the expected stoicheiometric relations are not strictly realized because other reactions, especially those catalysed by adenosine triphosphatase and adenylate kinase, introduce complicating factors. The steady incorporation of ^{32}P into the labile-phosphate fraction of the adenosine polyphosphate shows that ATP is continually formed, though its concentration does not rise.

The number of known substrate reactions which are coupled with the synthesis of ATP is small. Two occur in the course of the alcoholic and lactic acid fermentations of glucose and a third is the oxidative dephosphorylation of α-keto acids (see Krebs, 1953). The conversion of citrulline into ornithine is the fourth substrate reaction of this type to be described.

The decomposition of citrulline under the experimental conditions, i.e. on the acid side near the neutral point may be formulated as follows:

$$\underset{\text{(citrulline)}}{R.NH.CO.NH_2} + 2H^+ + H_2O \rightarrow \underset{\text{(ornithine)}}{R.NH_3^+} + NH_4^+ + CO_2.$$

The change of free energy of this reaction cannot be calculated owing to lack of basic data, but it is likely that it is not very different from that of the analogous reaction of urea

$$NH_2.CO.NH_2 + 2H^+ + H_2O \rightarrow 2NH_4^+ + CO_2. \quad (3)$$

The free energy of formation under standard conditions ($\Delta G°f$) of urea (solid) at 25° is −47·1 kcal. (Ruehrwein & Huffman, 1946). On the basis of the data of Borsook & Huffman (1938) for the calculation of $\Delta G°f$ of aqueous urea, a value of −48·7 is obtained. With this value, and the data for water, NH_4^+ (aq.), CO_2 (gas) given by Burton & Krebs (1953) ΔG of reaction (3) at 25° is −7·8 kcal. at pH 7 and −10·5 kcal. at pH 6. Under conditions more closely approximating to those of experiments (CO_2 pressure, 0·05 atm.; concentrations of urea and NH_4^+, 0·01 M), ΔG becomes considerably higher, namely −12·1 kcal. at pH 7 and −14·9 kcal. at pH 6. Under many physiological conditions, the concentration of NH_4^+ is likely to be much lower still, which would mean a higher ΔG value for reaction (3). These considerations indicate that sufficient energy is released in this type of reaction to make a synthesis of one pyrophosphate bond of ATP possible.

The coupling between the breakdown of citrulline and the synthesis of ATP appears to be obligatory, because citrulline is not broken down in the absence of phosphate and ADP, although arsenate can replace both ADP and phosphate (Knivett, 1954). It follows that unlike the hydrolysis of urea, the breakdown of citrulline is not a 'simple' hydrolysis.

SUMMARY

1. The phosphorylation of ADP to ATP coupled with the breakdown of citrulline to ornithine by a cell-free extract of *Streptococcus faecalis* has been studied quantitatively.

2. A mixture containing citrulline and ADP released CO_2, formed ATP, and removed ADP and inorganic phosphate, the four changes being approximately equivalent. The results agree with the view that the overall change is

$$\text{citrulline} + \text{ADP} + \text{phosphate} \rightarrow \text{ornithine} + CO_2 + NH_3 + \text{ATP}.$$

3. [^{32}P]Potassium dihydrogen phosphate was used to distinguish between ATP formed from ADP by the action of adenylate kinase (myokinase) and by synthesis involving inorganic phosphate. The appearance of ^{32}P in ADP, and in both of the labile phosphates of ATP, but not in AMP, indicates the presence of adenylate kinase in extracts of *Strep. faecalis*.

4. The fission of citrulline according to the reaction formulated in paragraph 2 represent a new type of coupled phosphorylation leading to a formation of pyrophosphate bonds of ATP.

I wish to thank Dr E. F. Gale, F.R.S., for his generous help and encouragement, and Prof. H. A. Krebs, F.R.S., for his helpful advice and interest in this work. I am grateful to Mr R. Hems for advice and technical assistance, to the Medical Research Council for a research training grant, and to the Director, Chemical Research Laboratory, Teddington, for permission to undertake this investigation.

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The Effect of Arsenate on Bacterial Citrulline Breakdown

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It has previously been shown (Knivett, 1952*a*, *b*; see also Slade, 1953, and Korzenovsky & Werkman, 1953) that arsenate increases the rate at which carbon dioxide is released from arginine and citrulline by enzyme preparations of *Streptococcus faecalis*. The present paper is a detailed study of the arsenate effect.

EXPERIMENTAL

The organism, growth medium, preparation of suspensions, and of the cell-free extract (prepared by the Mickle disintegrator), adenosine diphosphate (ADP) and triphosphate (ATP) were as previously described (Knivett, 1954).

Preparation of cell-free extracts. In addition to the extract prepared with the Mickle disintegrator (designated 'M' extract), a cell-free extract having a greater enzymic activity was prepared by crushing the cell-paste in the press described by Hughes (1951). The mucoid, crushed mass was taken up with water and centrifuged for 20 min. at 20000 *g* in a Sorvall high-speed angle head centrifuge (Ivan Sorvall Inc., New York). This extract is designated the 'P' extract.

Manometric methods. The evolution of CO_2 during citrulline breakdown was measured in Warburg manometers with conventional techniques. The activity of the washed cells was determined with arginine as substrate, as citrulline is attacked very slowly by intact cells. The rate of evolution is expressed as Q_{CO_2} (μl. CO_2 liberated/hr./mg. dry wt. of cells) calculated from the steady rate of gas output established after the first few minutes.

Experiments with cell-free extracts. Cell-free extracts were incubated at 37° with citrulline and a buffer of pH 5·8 (both sodium succinate and sodium arsenate buffers were used—see legends to tables); where necessary, CO_2 liberation was followed manometrically in parallel experiments. At suitable time intervals samples were removed and prepared for analysis. For the estimation of citrulline and ammonia a

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1·0 ml. sample of the reaction mixture was transferred to 4 ml. 0·25 N-HCl; 0·5 ml. of the acidified mixture was used for the estimation of citrulline and 1·0 ml. for the estimation of ammonia. For the estimation of ornithine a 2·0 ml. sample was pipetted into 0·5 ml. N-HCl, warmed to 80° for 5 min., transferred quantitatively to a manometer flask and the pH was adjusted to 5·8 with N-NaOH.

Estimation of ammonia. The Conway micro-diffusion method was used for the distillation of the ammonia (Conway, 1950) which was then estimated by nesslerization.

Estimation of ornithine. Ornithine was estimated by the specific decarboxylase method of Gale (1945). The organism used was a strain of *Escherichia coli* (EC4) kindly supplied by Dr H. D. Slade.

Estimation of citrulline. The carbamido/diacetyl reaction is a sensitive test for citrulline (Fearson, 1939; Gornall & Hunter, 1941; Archibald, 1944), but does not give very reliable quantitative results. Kawerau (1946) found that colour fading occurs owing to over-oxidation and could be avoided by the use of (*a*) a mild oxidizing agent (arsenious oxide in conc. HCl instead of potassium persulphate), (*b*) an excess of diacetyl monoxime, (*c*) a boiling time of 15 min. Under these conditions neither colour fading nor light sensitivity was noted in 30 min. On testing Kawerau's method, however, considerable differences in colour intensity were noted between different sets of estimations. This was traced to the variations in the time elapsing between mixing the reagents with citrulline and boiling the solutions to develop the colour. The amount of colour produced was approximately doubled if the reagents were allowed to stand overnight at room temperature before boiling. Previous workers have noted that the colour produced is not proportional to the concentration of citrulline and that standards must be included.

The calibration curves did not pass through the origin, but cut the abscissa at an intercept of approximately 0·2 μmole citrulline. Therefore 0·25 μmole citrulline was added to each determination to ensure that sufficient colour would be produced to be measurable, and a correction was applied. A typical calibration curve had a slope of log $I_0/I = 1{\cdot}040$ per μmole citrulline in 15 ml. at 492 mμ. with a 1 cm. light path. The method gave reliable results over a range 0–1·25 μmole. Reagents: 0·25 ml. 0·001 M L-citrulline; 3·75 ml. arsenious oxide (10%, w/v, in conc. HCl); 2·0 ml. diacetyl monoxime (1%, w/v, in 5%, v/v, acetic acid). The combined reagents (6·0 ml.) were added from a burette to 1·0 ml. of the test solution (containing not more than 1·25 μmoles citrulline) and kept overnight for the colour to develop. The covered tubes were heated in a boiling-water bath with frequent shaking for 15 min., cooled and distilled water was added to 15 ml. The colour produced was estimated in the Beckman Spectrophotometer at 492 mμ.; twelve estimations and four standards containing known amounts of citrulline were dealt with in a batch.

Estimation of protein. The protein content of the extracts was measured by the method of Kalckar (1947).

RESULTS

Effect of arsenate on the breakdown of arginine in washed bacterial suspensions. Table 1 illustrates the striking effect of arsenate on the rate of carbon dioxide formation from arginine by washed suspensions of *Strep. faecalis*. At a concentration of 0·33 M arsenate increased the rate more than fivefold and at 0·008 M about twofold. Phosphate (0·33 M) only increased the rate by about 25%. Addition of phosphate in the presence of arsenate reduced the stimulating effect of arsenate.

Effect of arsenate on the breakdown of citrulline in cell-free extracts. As previously shown (Knivett, 1954), citrulline is not attacked at an appreciable rate by cell-free extracts unless both ADP and inorganic phosphate are added. Table 2 shows that phosphate can be replaced by arsenate, and that the effect of higher concentrations of arsenate (0·016 M) is reduced by the addition of phosphate. In contrast to phosphate, arsenate stimulated the formation of carbon dioxide when no ADP has been added

Table 1. *The effect of arsenate and phosphate upon the release of* CO_2 *from arginine by washed suspensions of* Streptococcus faecalis

Total volume of fluid per Warburg cup, 3 ml. All cups contained 1·0 ml. M buffer (sodium phosphate, arsenate or phthalate), pH 5·8; 1·0 ml. of a washed suspension of cells (containing 2·34 mg. dry wt./ml.); and in the side compartment 0·5 ml. 0·03 M L-arginine HCl. Additions to the main compartment: 0·25 ml. 0·1 M sodium arsenate or sodium phosphate buffer, pH 5·8; water to volume. Gas: air. Temp. 37°.

	Final concentration of		
Main buffer	Phosphate (M)	Arsenate (M)	Q_{CO_2}
Phthalate	0	0	96
Phosphate	0·33	0	120
Arsenate	0	0·33	516
Phthalate	0·008	0	85
Phthalate	0	0·008	186
Phthalate	0·008	0·008	122

Table 2. *The effect of phosphate and arsenate upon the release of* CO_2 *from citrulline in a cell-free extract in the presence of ADP*

Total volume of fluid per cup, 3 ml. All cups contained 0·5 ml. 0·5 M potassium succinate buffer, pH 5·8; 0·1 ml. 0·12 M-$MgCl_2$; 0·9 ml. 0·008 M ADP; 0·6 ml. 0·3 M L-citrulline; and in the side compartment 0·3 ml. 'M' extract (35·2 mg. protein/ml.). Additions to the main compartment: varying quantities of 0·12 M sodium arsenate buffer, pH 5·8, and 0·18 M sodium phosphate buffer, pH 5·8; water to volume. Gas: air. Temp. 30°.

Expt. no.	Incubation time (min.)	Final concentration of Phosphate (M)	Final concentration of Arsenate (M)	CO_2 released (μmoles)
1	34	0	0	1·6
		0·006	0	8·1
		0	0·016	18·6
		0·006	0·016	12·6
		0·012	0·016	11·7
2	21	0	0	1·3
		0	0·004	7·2
		0	0·008	12·2
		0	0·016	16·3

(Table 3). This effect of arsenate is reduced by the addition of either phosphate, or fluoride, or iodoacetate.

The following experiment shows that the inhibition by phosphate is competitive. The rate of carbon dioxide production was measured over a range of arsenate concentrations, with and without phosphate. The reciprocal of the initial velocity was plotted against the reciprocal of arsenate concentration according to the method of Lineweaver & Burk (1934) and the line of closest fit calculated by the method of least squares (Fig. 1). The Michaelis constant (K_m) was 0·022 M in the absence of added phosphate. When phosphate (0·0067 M) was added, the apparent Michaelis constant was 0·093 M. The fourfold increase of apparent Michaelis constant on the addition of phosphate indicates that the inhibition is competitive. The rate of carbon dioxide release was measured over a range of citrulline concentrations (0–0·04 M) (Fig. 2). The Michaelis constant for citrulline in the presence of 0·029 M arsenate was 0·12 M.

Table 3. *Release of CO_2 from citrulline in a cell-free extract in the presence of arsenate (no adenosine phosphate added)*

Total volume of fluid per cup, 3 ml. All cups contained 0·5 ml. 0·5 M potassium succinate buffer, pH 5·8; 0·1 ml. 0·12 M-$MgCl_2$; 0·3 ml. 'M' extract (11·7 mg. protein/ml.), and in the side compartment 0·6 ml. 0·3 M L-citrulline. Other reagents added to side compartment. Gas: air. Temp. 30°. Incubation time 1 hr.

Additions	CO_2 released (μmoles)
None	0·0
Arsenate (0·004 M)	7·9
Arsenate (0·008 M)	12·0
Arsenate (0·008 M); phosphate (0·012 M)	3·6
Arsenate (0·008 M); NaF (0·01 M)	3·2
Arsenate (0·008 M); iodoacetate (0·01 M)	8·2

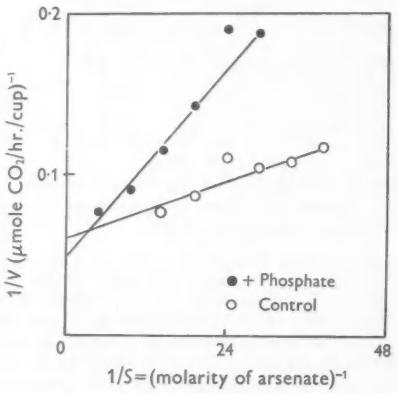

Fig. 1. Competitive inhibition by phosphate of the release of CO_2 from citrulline in the presence of arsenate. Total volume of fluid per cup, 1·5 ml. Main compartment: 0·3 ml. 0·5 M potassium succinate buffer, pH 5·8; 0·3 ml. 0·3 M L-citrulline; 0·5 ml. sodium arsenate buffer (0·026–0·208 M), pH 5·8; 0·1 ml. 0·1 M potassium phosphate buffer, pH 5·8, or water; and in the side compartment: 0·3 ml. of a mixture of 1·3 ml. 0·12 M-$MgCl_2$, 2·4 ml. 0·5 M potassium succinate buffer pH 5·8, and 0·2 ml. 'P' extract (4·6 mg. protein/ml.). Gas: air. Temp. 37°. CO_2 release was measured at 5 min. intervals after tipping, and the velocity calculated from the steady gas output in the first 20 min.

Products of citrulline breakdown in the presence of arsenate. Estimation of the amount of citrulline disappearing and the amount of ornithine, ammonia and carbon dioxide formed shows that the changes in the amounts of the four reagents are approximately equivalent (Table 4). The overall reaction of citrulline in the presence of arsenate is thus the same as in the presence of ADP and phosphate. The breakdown of citrulline was very rapid at first, but as would be expected from the high values of K_m for arsenate and citrulline, the rate of the reaction slowed down as the concentration of citrulline fell.

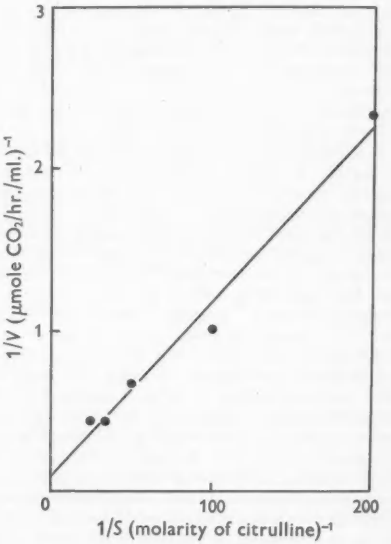

Fig. 2. The effect of citrulline concentration on the rate of release of CO_2 from citrulline in the presence of arsenate. Total volume of fluid per cup, 3 ml. All cups contained 1·0 ml. 0·5 M potassium succinate buffer, pH 5·8; 0·1 ml. 0·12 M-$MgCl_2$; 0·1 ml. 0·875 M sodium arsenate buffer, pH 5·8; 0·5 ml. 'P' extract (0·3 mg. protein/ml.); and in the side compartment varying quantities of 0·3 M L-citrulline, and water to volume. Gas: air. Temp. 37°. The rate of CO_2 evolution was constant during the first 100 min.

Table 4. *The products of citrulline breakdown in the presence of arsenate*

18 ml. M sodium arsenate buffer pH 5·8, 3·6 ml. 'P' extract, 3·6 ml. 0·12 M-$MgCl_2$, and 10·8 ml. 0·33 M L-citrulline. The reaction started after thermal equilibration by the addition of citrulline. Temp. 37°. For analytical procedures see methods. The ornithine samples were taken 0·5 min. later than the citrulline and ammonia samples. Quantities are corrected for control without citrulline.

Time (min.)	CO_2 released (μmoles/ml.)	NH_3 formed (μmoles/ml.)	Ornithine formed (μmoles/ml.)	Citrulline found (μmoles/ml.)	Citrulline removed (μmoles/ml.)
6·5	1·50	1·46	1·50	7·96	2·04
18·5	3·50	3·41	3·48	6·36	3·64
25·5	4·35	4·35	4·05	5·71	4·29
33·0	5·15	5·39	5·04	4·84	5·16
85·0	8·00	—	7·99	1·88	8·12
150·0	9·36	9·40	8·99	0·36	9·64
		Control without citrulline			
150·0	0·0	0·55	0·52	0·44	—

DISCUSSION

Accelerating effects of arsenate on enzymic reactions were first described by Harden & Young (1911), who found an increased rate of alcoholic fermentation in yeast extract. Similar effects have been observed by Needham & Pillai (1937) and Warburg & Christian (1939) in the phosphoglyceraldehyde dehydrogenase system (see also Bücher & Garbade, 1952*a*, *b*; Racker & Krimsky, 1952), by Doudoroff, Barker & Hassid (1947) in the sucrose phosphorylase system and by Stadtman & Barker (1950), see also Stadtman, Novelli & Lipmann (1951) in the transacetylase system, by Delwiche, Loomis & Stumpf (1951) in the glutamyl transphorase system, and by Kennedy & Barker (1951) in the fatty acid oxidation system. A common feature of the arsenate effects is that they occur in systems in which phosphorylated intermediates participate. Following the view of Warburg & Christian (1939) an effect of arsenate is taken to be due to the formation of an arsenate analogue of the phosphorylated intermediate which differs from the phosphate derivative in that it is readily hydrolysed.

The observations on the citrulline system are in accordance with this general concept of the action of arsenate. Thus at least two enzymes must be concerned with the decomposition of citrulline: (1) an enzyme bringing about the formation of a phosphorylated intermediary, possibly phosphocitrulline, (2) a phosphokinase which transfers phosphate from the phosphorylated intermediary to ADP. It must further be assumed that this compound is relatively stable in the absence of ADP and that the transfer of phosphate to ADP is coupled with the release of carbon dioxide and ammonia, also that the arsenate compound is unstable and, unlike the phosphorylated compound, decomposes spontaneously into ornithine, carbon dioxide, ammonia and arsenate. Since attempts to demonstrate phosphocitrulline in the reaction mixture have been unsuccessful, if it occurs at all its concentration must be very low; and the equilibrium in the system

$$\text{citrulline} + \text{phosphate} \rightleftharpoons \text{phosphocitrulline}$$

must be unfavourable for the accumulation of phosphocitrulline.

SUMMARY

1. The rate of the conversion of citrulline into ornithine, carbon dioxide and ammonia by a cell-free extract of *Streptococcus faecalis* in the presence of phosphate and adenosine diphosphate (ADP) is increased by the addition of arsenate.

2. In the presence of arsenate, citrulline is decomposed by extract without the addition of phosphate and ADP. The maximum rate in the presence of arsenate is about 5 times higher than that in the presence of ADP and phosphate.

3. The products formed from citrulline are the same with arsenate as with ADP and phosphate; the overall reaction in the presence of arsenate being

$$\text{citrulline} + H_2O = \text{ornithine} + CO_2 + NH_3.$$

4. The effect of arsenate is reduced by the addition of phosphate, or fluoride, or iodoacetate. The inhibition by phosphate is competitive.

5. The mechanism of the action of arsenate is discussed.

6. An improved method for the estimation of citrulline is described; it is based on the diacetyl reaction of Fearon (1939).

I wish to thank Dr E. F. Gale, F.R.S., for his generous help and encouragement, and Prof. H. A. Krebs, F.R.S., for his helpful advice and interest in this work. I am indebted to the Medical Research Council for a research training grant, and to the Director, Chemical Research Laboratory, Teddington, for permission to undertake this investigation.

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Interaction of Acids of High Molecular Weight with Albumin

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Sodium deoxyribonucleate has been shown to prevent the coagulation by heat of dialysed albumin solutions at about pH 5·5 (Carter & Greenstein, 1946; Greenstein & Hoyer, 1950). Evidence of such interactions between nucleic acids and proteins is of considerable interest, since one of the main problems of nucleoprotein structure is the way in which the protein and nucleic acid are bound together. In the experiments described here it is shown that polyacids in general are able to interact with albumin so as to prevent heat coagulation. A short account of this work has already been presented (Hamer, 1953*a*).

EXPERIMENTAL

Albumin solutions. Bovine plasma albumin (fraction V, Armour and Co.) was used throughout this work. A solution was made up containing approx. 2 g./100 ml. and the pH was adjusted to 6·7–6·8. The solution was then dialysed using cellulose casings (Visking Corp., Chicago) at 2–4° against distilled water. After 4 or 5 days the pH dropped to about 5·5–5·7 and the final protein concentration was then 1·4 g./100 ml., as estimated from the N content.

Other materials. Deoxyribonucleic acids were prepared from isolated calf-thymus nuclei: specimen 1 by saline extraction followed by treatment of the solution with chloroform:butanol mixtures and specimen 2 by saline extractions followed by deproteinization with sodium dodecyl sulphate (Hamer, 1953*b*). A commercial sample of yeast ribonucleic acid was used (British Drug Houses Ltd.). Heparin and alginic acid were also obtained from commercial sources. The heparin had an activity of 90 i.u./mg. (Evans, Lescher and Webb Ltd., batch N 10090) and a molecular weight of probably 17000 (Jensen, Snellman & Sylven, 1948). The alginic acid had a molecular-weight range of 10000–20000 (specimen HS/LD and details, including the mol.wt. which is based on viscosity determinations, supplied by Alginate Industries Ltd.). Dextran sulphate and dextran were supplied by Dr K. Walton. Both had molecular weights about 8000, as determined by measurements of osmotic pressure and intrinsic viscosity (see Walton, 1952; Ricketts, 1952). Polyglutamic acid was prepared from the capsular substance of *Bacillus subtilis* by the method of Hanby & Rydon (1946), the final product being dialysed and freeze-dried (supplied by F. W. Moore, Bacteriology Department, Birmingham University). The molecular weight of material obtained by this method was reported to be at least 15000.

Aqueous solutions (0·1 %) of these compounds were prepared; where necessary to neutralize alginic and nucleic acids a trace of alkali was added and the solutions were dialysed in the cold to remove salts. It may be noted here that the amounts of these solutions used in the tests described below were so small as to have no effect on the pH of the albumin solutions.

Heat coagulation. 2 ml. of albumin solution with 2 ml. of water (or 2 ml. of the solution under investigation) were heated in a boiling-water bath for 10 min. and chilled in cold water. Under these conditions the albumin coagulates to give a milky-white, colloidal solution which is quite stable

and does not flocculate on standing. Turbidities of the solutions were measured on the Hilger Spekker Absorptiometer using neutral glass filters (H508) and 2·5 mm. cells. The turbidities of protected solutions were expressed as percentages of the value obtained for an unprotected (0·70%) solution.

Optical rotation. Measurements were made with a Hilger Standard Polarimeter using sodium light and a 1 dm. tube. Tests were carried out on 0·7 and 1·28% (w/v) albumin solutions protected with sufficient dextran sulphate or polyglutamate to prevent any opalescence developing on heating. The rotation of these solutions was determined before and after heating and also after the addition of guanidine hydrochloride to final concentration 3·55 M.

Metachromatic tests. Mixtures of the same composition as those used in the coagulation experiments were tested with a 1% solution of toluidine blue.

RESULTS

The results obtained for two different specimens of deoxyribonucleate confirm the findings of Greenstein and co-workers (Fig. 1). Percentage turbidity of the protected solution is plotted against the final concentration of nucleic acid phosphorus and it will be seen that 3–4 μg. are sufficient almost completely to prevent the heat coagulation of the albumin. The weight ratio of albumin to nucleate would be about 200:1 at this point. However, contrary to the results of Carter & Greenstein (1946), it was found that yeast ribonucleate had a comparable effect, inhibiting coagulation to about the same extent as specimen 2 (Fig. 1). This difference may be due to the fact that the experiments of Carter & Greenstein were carried out with liver-tissue extracts and not albumin solutions.

To examine whether the protective effect was only characteristic of polyphosphoric acid groupings, heparin and dextran sulphate were tested with albumin in the same way. These compounds, which may be considered as polysulphates, behaved in the same way as did the nucleates (Fig. 2). Also, though the molecular weight of heparin is about 17000 and that of dextran sulphate about 8000, weight for weight both compounds had the same effect on the albumin coagulation. Complete protection was given by about 1 part of the polysulphate to 300 parts of albumin. A dextran of corresponding molecular weight had no effect, showing that the presence of the acidic groups was essential for the protection of the albumin.

Finally, several polycarboxylic acids were tested. Fig. 3 shows the results obtained with alginate and polyglutamate. Once more a protective effect was observed. This was not quite so strong as with the

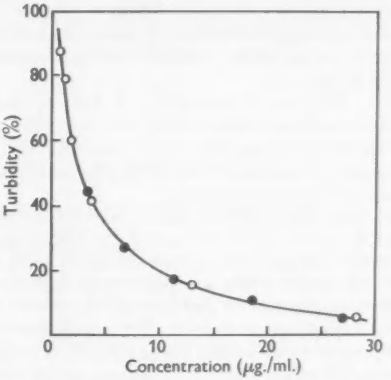

Fig. 2. Effect of heparin and dextran sulphate on the heat coagulation of albumin solutions. Albumin concn., 0·70%. ○, heparin; ●, dextran sulphate.

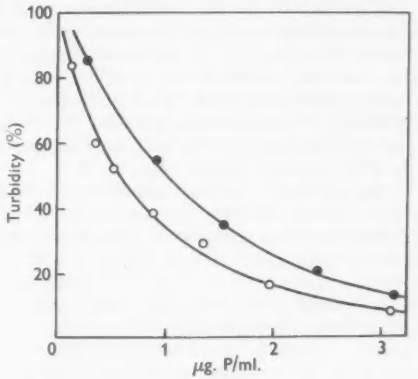

Fig. 1. Effect of deoxyribonucleate specimens on the heat coagulation of albumin solutions. Albumin concn., 0·70%. ○, specimen 1; ●, specimen 2.

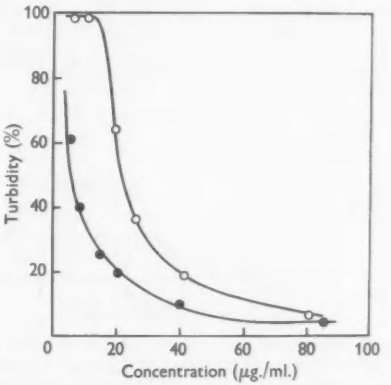

Fig. 3. Effect of polyglutamate and alginate on the heat coagulation of albumin solutions. Albumin concn., 0·70%. ○, alginate; ●, polyglutamate.

poly-sulphates and -phosphates, coagulation being prevented by 1 part of polycarboxylate per 100 parts of albumin. Hyaluronate was also effective. It may be added that in all cases plots of percentage turbidity against the logarithm of the polyacid concentration were linear, except at very high and very low turbidities.

Prevention of the heat coagulation of albumin was only obtained in the absence of salts. As the concentration of salt in the solution was increased, the protective effect was lowered and the turbidity of the solution increased. Fig. 4 illustrates a case where sodium chloride was added in increasing amounts to albumin solutions protected with dextran sulphate. When a point was reached at which the salt concentration (by weight) was about 10 times that of the dextran sulphate, a solid mass of coagulum was formed. Addition of salt to a heated protected solution of albumin does not, however, bring about precipitation or flocculation of the protein.

The following compounds of low molecular weight, used as sodium salts, had no effect at a similar concentration (by weight) and pH: glycerophosphate, guanylate, 2-naphthol-3:6-disulphonate, glucuronate and glutamate.

Considerable evidence was put forward by Greenstein & Hoyer (1950) to show that on heating, the protected albumin was in fact denatured, but that the interaction with the deoxyribonucleate prevented coagulation of the denatured protein. It is probable that a similar process occurs with the other compounds studied here. Some additional evidence has been found for albumin solutions protected with dextran sulphate or polyglutamate, by following the optical rotation. On heating, there was an increase in $[\alpha]_D^{21}$ from $-60{\cdot}4°$ to $-72{\cdot}9°$ (independent of the protecting agent and the albumin concentration), showing that some molecular rearrangement had occurred. Jirgensons (1952) has shown that optical-rotation changes are a very sensitive guide to denaturation in albumin and other proteins. Possibly the change occurring in the molecule is more limited than, or of different character from, that caused by guanidine hydrochloride, where extensive denaturation is produced. Addition of guanidine hydrochloride to these heated solutions produced an additional change in $[\alpha]_D^{21}$ to $-100{\cdot}3°$. This is comparable with the rotation of an unprotected albumin solution similarly denatured with guanidine hydrochloride, which was found to be $-102°$.

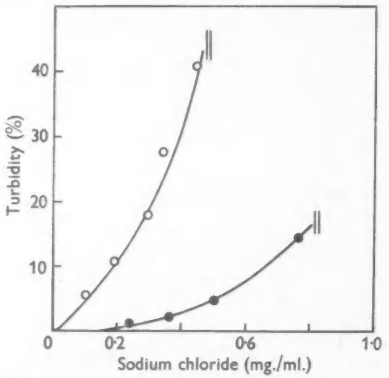

Fig. 4. Effect of increasing concentration of NaCl on the heat coagulation of albumin solutions protected with dextran sulphate. ○, albumin (7·0 mg./ml.) and dextran sulphate (0·05 mg./ml.); ●, albumin (7·0 mg./ml.) and dextran sulphate (0·10 mg./ml.). ‖ indicates coarse coagulation.

The polysulphates and polycarboxylates studied gave the characteristic red-purple metachromatic reaction with toluidine blue. In the presence of albumin, both before and after heating, no such colour change was observed, indicating that an interaction exists between the protein and the polyacid.

DISCUSSION

In summarizing the experimental observations given above, it may be said that polyacids of molecular weight from about 8000 upwards are able to interact with serum albumin at pH about 5·5 in salt-free solution so as to prevent the coagulation of the albumin when heated. One part by weight of the polyacid is sufficient to prevent the heat coagulation of 100–300 parts of albumin. If one considers a mechanism of denaturation of the type proposed by Haurowitz (1950), then one could picture that, although intramolecular salt bridges in the albumin molecules are broken down by heating, the interaction already existing between the protein and the acid prevents reorientation of the albumin molecules. By such action, formation of intermolecular salt bridges, and hence coagulation, would be prevented. Recently Cheesman (1953) has found evidence of interaction of deoxyribonucleic and ribonucleic acids with proteins by observing the reorientation of protein dipoles in surface films.

It has not been possible at present to obtain a definite relation between the amount of albumin and the character and amount of polyacid necessary to prevent coagulation. The molecular weight of the protecting agent does not seem important, since similar effects were obtained with compounds varying from dextran sulphate (8000) to deoxyribonucleate (probably $1–2 \times 10^6$). However, heat coagulation is prevented when each molecule is balanced by one to two acid groups of nucleic acid, about two acid groups of dextran sulphate and three to four groups of alginate or polyglutamate. There

may well be some additional steric and ionization effects to be considered which would account for these differences in equivalence of acid groups and albumin molecules.

SUMMARY

1. Polyacids interact with albumin in salt-free solutions to prevent coagulation of the protein by heat. Polyphosphates (nucleic acids), polysulphates (heparin and dextran sulphate) and polycarboxylates (alginic acid and bacterial polyglutamic acid) were all effective. Effects on optical rotation of the albumin were also demonstrated.

2. The protecting agent acts by preventing the coagulation of the albumin, although denaturation occurs to some extent. The molecular size of the protecting agent seems of less importance than the number of acid groups in determining the degree of protection.

This work was carried out in the laboratories of the Birmingham branch of the British Empire Cancer Campaign.

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Phospholipids

2. ESTIMATION OF AMINO NITROGEN IN INTACT PHOSPHOLIPIDS

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During the course of work on the separation of phospholipids by chromatographic procedures the need became apparent for a rapid micromethod of detecting and estimating amino-containing phospholipids, particularly phosphatidylethanolamine, in the presence of much larger quantities of lecithin (phosphatidylcholine)—if possible without prior hydrolysis of the phospholipid.

The procedure previously employed involves a lengthy hydrolysis with acid or alkali followed by removal of the liberated fatty acids and estimation of amino nitrogen in the water-soluble portion of the hydrolysate, usually by the Van Slyke method. Direct application of the Van Slyke method to the unhydrolysed phospholipid has been attempted but is subject to serious errors and requires a comparatively large quantity of material. Ninhydrin has been used qualitatively for the detection of free amino-containing phospholipids on paper chromatograms by Hecht & Mink (1952), Hack (1953) and others.

It has been suggested (Lea & Rhodes, 1953) that ninhydrin in the buffered methyl cellosolve reagent used by Moore & Stein (1948) for the estimation of amino acids, and by Levine & Chargaff (1951) for the estimation of nitrogenous constituents in lipid hydrolysates, might be applicable to the determination of unhydrolysed amino-containing phospholipids. The present paper describes this application and records some observations on the behaviour of phospholipids and of simpler fatty esters in the direct Van Slyke determination.

EXPERIMENTAL

Materials

Egg phospholipid. Crude hen's egg phospholipid was prepared by extraction of the freeze-dried yolk with methanol:chloroform (1:1, v/v) followed by evaporation, solution in chloroform, filtration and precipitation several times from ether at 0–3° with acetone (4 vol.). Contaminating, non-phospholipid substances containing amino N (particularly amino acids) were removed by passage in chloroform:ethanol solution through a cellulose column (Lea & Rhodes, 1953).

'Pure' lecithin. Phosphatidylcholine, prepared from egg phospholipid by the chromatographic (alumina column) method of Hanahan, Turner & Jayko (1951), contained no amino N detectable by the Van Slyke method after hydrolysis, and possessed an atomic N:P ratio of 1·01. As shown

below, it was still contaminated by a trace of amino-containing material which could be estimated by the more sensitive ninhydrin method.

The ninhydrin method

Procedure. The solvent, usually ethanol or chloroform, is rapidly removed at a low temperature from the phospholipid samples in $5 \times \frac{3}{8}$ in. test tubes, conveniently by use of a simple, rotary-film, vacuum evaporator (Lea, Hannan & Rhodes, 1951), and the residue taken up in 1 ml. methyl cellosolve, in which it readily dissolves. 1 ml. of the ninhydrin:citrate:stannous chloride in 50% (v/v) methyl cellosolve reagent of Moore & Stein (1948) is added and, on shaking, a cloudy solution is obtained. The tubes are fitted with slip-on aluminium caps and heated in a boiling-water bath for 20 min. After being cooled, 5 ml. methyl cellosolve are added and the optical density at 570 mμ. measured in a 1 cm. cell, using a Unicam Grating Spectrophotometer or other suitable instrument.

Methyl cellosolve is used as a diluent in place of the 50% aqueous propanol of the original method, and it has been found that 5 ml. will dissolve a sufficient quantity of egg phospholipid or its fractions to permit the detection of less than 0·1% of amino-containing phospholipid. The absorption spectrum of the colour developed with phosphatidylethanolamine agrees closely with that of dioxohydrindylidene:dioxohydrindamine and with those developed by several amino acids as given by Moore & Stein (1948). It was found that the $SnCl_2$ content of the reagent could be increased 2–3 times without affecting the optical density, but increasing the time of heating to 40 and 60 min. progressively decreased the intensity of the colour.

Egg phospholipid. The amino N content of this material, as determined by the Van Slyke manometric procedure after hydrolysis with 6 N-HCl in a sealed tube at 100° for 15 hr. and removal of the fatty acids, was of the order of 18% of the total N (as compared with approx. 20% before purification on cellulose), and extinction coefficients were plotted against amino N (as in Fig. 1). No serine could be detected by paper chromatography of the hydrolysate.

A small sample of synthetic α-palmitoyl-α′-linoleoylphosphatidylethanolamine, made available through the courtesy of Dr W. G. Rose, also showed a linear relationship between extinction coefficient and P content and a molar colour yield close to that of the egg phospholipid. Initial difficulties with reproducibility discussed below, however, caused supplies of the synthetic material to become exhausted before an accurate comparison of the colour yields of the natural and synthetic products could be obtained.

Lecithin. The colour developed by 'pure' lecithin with the ninhydrin reagent was relatively very weak (0·074% of the phosphatidylethanolamine reaction) and was almost certainly due to residual traces of amino-containing impurities in the preparation rather than to any ninhydrin reaction of phosphatidylcholine itself (Fig. 1).

Phosphatidylethanolamine in presence of excess of lecithin. To test for possible interference by lecithin when present in large excess, three dilutions of egg phospholipid in 'pure' lecithin were prepared and examined by the ninhydrin method. When corrected for the small contributions due to the added lecithin the colour measurements showed no evidence of any interference by excess of lecithin in the estimation of phosphatidylethanolamine by this method (Table 1).

Ethanolamine. The ninhydrin method in this form can also be used for the estimation of ethanolamine (plus serine, if present) in a phospholipid hydrolysate and requires much less material than the Van Slyke method (Fig. 1). The recrystallized ethanolamine hydrochloride used gave an amino N:total N ratio of 1·00 by the Van Slyke procedure with a reaction time of 4 or 10 min. Levine & Chargaff (1951) have reported data (in arbitrary units) for ethanolamine and serine by the original Moore & Stein procedure.

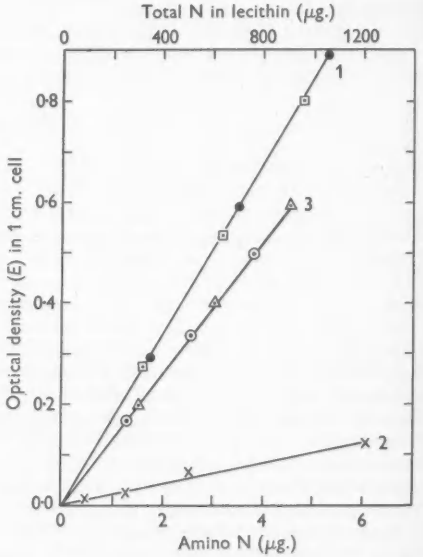

Fig. 1. Ninhydrin reaction of unhydrolysed phospholipids and of ethanolamine. Curve 1: egg phospholipid; curve 2: 'pure' egg lecithin; curve 3: ethanolamine hydrochloride.

Table 1. *Determination of phosphatidylethanolamine in presence of excess of lecithin*

Phosphatidylethanolamine N in sample (μg.)	*E* values* at various concentrations (%) of phosphatidylethanolamine N/total N				
	19·6	4·67	0·96	0·45	Av.
1·16	0·170	0·158	0·150	0·161	0·160
2·32	0·306	0·288	0·288	0·281	0·291
3·45	0·454	0·453	0·448	0·433	0·448
4·62	0·603	0·582	0·629	0·627	0·610
5·78	0·760	—	0·760	—	0·760

* Corrected for the small absorption of the 'pure' lecithin used in making the dilutions.

Influence of variables in the preparation and storage of the ninhydrin reagents. Moore & Stein (1948) state that, if only a few amino acid analyses are being run at one time, the necessary small quantity of ninhydrin reagent can be prepared for immediate use and run in from a burette. Alternatively, when large numbers of samples are being run the ninhydrin solution can be stored under N_2 in a brown- or red-glass reservoir attached to a pipetting machine.

In the first part of the present work small, freshly prepared batches of ninhydrin reagent were used, and excellent linear calibrations of optical density against amount of phospholipid amino N consistently obtained, but the slope of the curves, i.e. the molar colour yield tended to vary erratically from one run to another. The values recorded in Table 1 were obtained at an early stage of the investigation by the small-batch method and, although consistent within themselves, are approximately 20% lower than those subsequently obtained (Fig. 1) by the method described below using, however, new stock preparations of recrystallized ninhydrin and redistilled methyl cellosolve.

For all later determinations the ninhydrin reagent was prepared in 1 l. batches, de-aerated and stored under N_2 with protection from light. Under these circumstances variability was greatly reduced and the results did not seem to change appreciably with the age of the batch of reagent over a period of a few weeks, nor was there any appreciable difference between several batches of reagent prepared from the same stock of ninhydrin and redistilled methyl cellosolve. One batch of reagent saturated with hydrindantin, the reduced form of ninhydrin, was also prepared according to Fowden (1951) and gave similar results. Data illustrating these points are shown in Table 2 for egg phospholipid and for ethanolamine.

The presence of impurity, presumably peroxide, in the methyl cellosolve used as solvent for the phospholipid could be an important factor in producing erratic results. Moore & Stein (1948) did not describe any purification of the methyl cellosolve used in preparation of their ninhydrin reagent but stated that it gave a negative or very faint positive test with 10% (w/v) aqueous KI. Fowden (1951), in the estimation of amino acids eluted from paper chromatograms, specified redistilled methyl cellosolve shown to be free from oxidizing agents by the starch:iodide test. In our experience, redistilled methyl cellosolve exposed to diffused daylight can regenerate sufficient peroxide during 1 week to give a reduction in colour yield of 15%, but is comparatively stable in the dark (Table 2).

The Van Slyke method

Intact phospholipids. Chargaff, Ziff & Rittenberg (1942) used 5 min. Van Slyke values as a direct measure of the amino-N content of phospholipids and similar methods have been used by others. Unhydrolysed phospholipids, however, do not give satisfactory values in the manometric Van Slyke amino-N determination but continue to evolve N_2 with increasing time of reaction (Fig. 2, curve 1). Further indication of an abnormal reaction was given in this case by the production of a gas (possibly N_2O) which was incompletely absorbed by the alkaline permanganate in the Hempel pipette but which dissolved on further shaking with water or dilute acetic acid in the reaction chamber; reproducible values were then obtained.

'Pure' lecithin also produced an appreciable evolution of gas in the Van Slyke determination (Fig. 2, curve 2) and the similar shapes of the lecithin- and egg-phospholipid curves suggested that the slowly rising portion at least was due to some factor common to both systems, probably the unsaturated fatty acids. Correction of curve 1 by curve 2 gave a fairly constant value (curve 3) which was only slightly

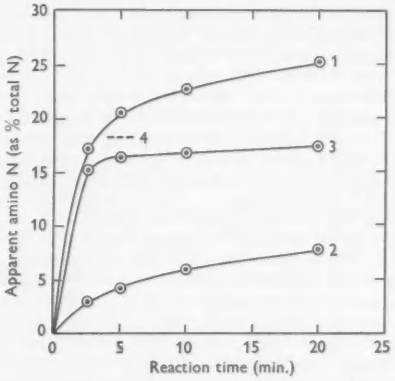

Fig. 2. Behaviour of unhydrolysed phospholipids in the Van Slyke determination. Curve 1: egg phospholipid; curve 2: egg lecithin; curve 3: subtraction of curve 2 from curve 1; curve 4: acid-hydrolysed egg phospholipid after removal of fatty acids.

Table 2. *Effect of variation in reagents on molar colour yield*

Ninhydrin reagent	Cellosolve diluent	$10^{-3}\epsilon$
Egg phospholipid		
Batch *A*, fresh	Freshly distilled	164
Batch *B*, fresh	Freshly distilled	163
Batch *C*, 10 weeks old	Freshly distilled	164
Batch *C*, 11 weeks old	Freshly distilled	163
Batch *C*, 11 weeks old	6 months old (in dark)	162
Batch *C*, 10 weeks old	1 week old (in light)	139
Fresh, with hydrindantin	Freshly distilled	162
Ethanolamine		
Batch *B*, fresh	Freshly distilled	127
Batch *C*, 10 weeks old	Freshly distilled	128
Fresh, with hydrindantin	Freshly distilled	126

lower than the value obtained by Van Slyke determination on the acid hydrolysate after removal of the fatty acids (Fig. 2, curve 4), but such a correction could only be completely effective if the fatty acid compositions of the two systems were identical, which in this case they were not.

A reaction time, in this case 3 min., can be chosen empirically from curve 1 to give the value found after hydrolysis, but such a result will depend in some degree on the composition of the fatty acid mixture present. Kline, Gegg & Sonoda (1951) have made some use, with unhydrolysed egg phospholipid, of a 5 min. reaction period which they found to correspond to the value obtained after hydrolysis for 48 hr. with 2N-H_2SO_4. Kirk, Page & Van Slyke (1934) used three times the 'normal' reaction time of 198 sec. at 23·6° for 'complete' reaction with plasma lipids, but apparently did not calibrate against a hydrolysate. Subsequently, Folch, Schneider & Van Slyke (1940) attributed high values on intact lipids to the presence of unsaturated fatty acids, since hydrogenation greatly reduced the error, but later Folch (1942) reported low and inconsistent results on phosphatidylethanolamine from brain and attributed them to coagulation of the emulsion in the nitrous acid reagent whereby the phospholipid became partly inaccessible to attack. In view of these conflicting observations it seemed advisable to examine the behaviour of a series of simpler fatty esters.

Fatty esters. 'Pure' egg lecithin, the methyl esters prepared from its constituent fatty acids, pure ethyl palmitate (used because it has a lower m.p. than the methyl ester), methyl oleate, linoleate and linolenate and a highly unsaturated methyl ester fraction obtained by crystallization of linseed-oil esters at −60° were all treated with nitrous acid for 5 min. under the conditions of the Van Slyke determination, with the addition of a drop of 'Teepol' (sodium alkyl sulphate, Shell Co. Ltd.) to ensure adequate dispersion of the insoluble esters. The result (Fig. 3) showed an apparent amino-N content which was negligible for the saturated ester but which increased rapidly with increasing unsaturation, especially after the first double bond. Since the value for unhydrolysed lecithin falls on the same curve as those for the simple esters, the high value obtained by direct application of the Van Slyke method to the unhydrolysed phospholipid can be accounted for by its content of unsaturated fatty acids.

Hydrolysis procedures. Many different methods have been used for the hydrolysis of phospholipids prior to estimation of their nitrogenous constituents. In our experience methanolysis by refluxing with 6N-HCl in dry methanol for 3 hr. followed by refluxing with dilute aqueous HCl for 1 hr. (to hydrolyse any serine ester which might be present) and extraction with light petroleum to remove fatty acids gave amino-N values about 9% lower than hydrolysis with aqueous 6N-HCl at 100° for 25 hr. followed by chilling and separation of the fatty acids by filtration. This is in agreement with the observations of Levine & Chargaff (1951) who obtained lower recoveries of N and choline from egg lecithin by methanolysis (79·5%) than by aqueous hydrolysis for 48 hr. (98·2%), although in the same paper (their Table VII) they obtained with a series of brain lipid fractions approximately the same recoveries of N by the two methods.

The fatty-acid residues can readily be split off from glycerophospholipids by acid or (particularly) alkali, and the residual glycerylphosphorylcholine and presumably (by analogy) glycerylphosphorylethanolamine are further decomposed quite readily into glycerophosphoric acid and the nitrogenous base. For example, N-HCl at 97° will completely remove choline from glycerylphosphorylcholine in less than 30 min. (Schmidt, Hershman & Thannhauser, 1945). Phosphorylcholine and phosphorylethanolamine, however, are very much more resistant to hydrolysis. Baer & McArthur (1944) found only 20% splitting of phosphorylcholine by N-HCl at 100° after 12 hr. and our own results, confirming those of Levine & Chargaff (1951), indicate only 95–98% hydrolysis of phosphorylethanolamine (prepared by the method of Outhouse, 1937) by 6N-HCl in 48 hr. at 100°. These latter substances, however, are probably not to any great extent intermediates in the hydrolysis of glycerophospholipids by acid or alkali.

As a check on the rate of hydrolysis of the mixed egg phospholipids, a specimen, purified by rejection of material insoluble in cold ether and in 95% ethanol, by passage through a cellulose column and by dialysis to an atomic N:P ratio of 1·00, was hydrolysed with 6N-HCl in sealed ampoules under nitrogen for 3–24 hr. The fatty acids were then removed by filtration and extraction with light

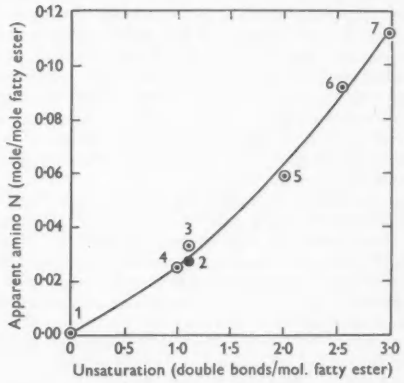

Fig. 3. Apparent amino-N content of fatty esters by the Van Slyke manometric method (5 min. with nitrous acid at 20°) as a function of unsaturation. (1) Ethyl palmitate; (2) egg lecithin; (3) methyl esters of fatty acids of (2), iodine value (I.V.) 94, mean mol.wt. 300; (4) methyl oleate, I.V. 86, mol.wt. 296; (5) methyl linoleate, I.V. 173, mol.wt. 294; (6) methyl ester fraction from linseed oil, I.V. 220, mean mol.wt. 293; (7) methyl linolenate, I.V. 260, mol.wt. 292.

Table 3. *Hydrolysis of egg phospholipid by* 6N-HCl *at* 100°

Time at 100° (hr.)	Amino N*	Choline N*	Amino N + choline N*
3	19·2	80·4	99·6
6	18·7	81·6	100·3
12	18·7	78·5	97·2
24	18·8	82·3	101·1
Average	18·9	80·7	99·6

* As % of the total N recovered.

petroleum and the aqueous phase was analysed for total N, amino N and choline (as reineckate by the method of Entenman, Taurog & Chaikoff, 1944). Approximately 98% of the original N was recovered in the aqueous phase after 3 hr., i.e. a complete recovery within experimental error and all of the choline was already free (Table 3). Apparently the very long periods of hydrolysis often employed are not necessary if the aim is only to estimate fatty acids, amino N and choline N in glycerophospholipids.

DISCUSSION

The ninhydrin method. The results presented in this paper indicate that the ninhydrin procedure of Moore & Stein (1948), modified to maintain the lipids in solution, can be used as a rapid and sensitive method for the estimation of phosphatidylethanolamine in the presence of much larger quantities of non-amino phospholipids without the necessity for previous hydrolysis. The behaviour of phosphatidylserine has not been examined but there is no reason to believe that this substance could not similarly be estimated.

Strict conformity with Beer's law and satisfactory reproducibility were easily obtained within any one run, but considerably different molar colour yields could result in different runs with the same substance unless care was taken with the purification and storage of the reagents and particularly with the elimination of peroxide from the cellosolve used as solvent for the phospholipids. As with any technique involving a ninhydrin reagent, it is desirable to carry out check determinations at intervals with one or more concentrations of a standard substance. The standard chosen should preferably be of the same type as the material under examination in view of the rather surprising observation of Levine & Chargaff (1951) that the molar colour yields from serine and ethanolamine when determined with different ninhydrin solutions by the normal Moore & Stein procedure did not maintain a constant relationship to one another, but fluctuated widely. Using seven different batches of reagent, apparently stored under nitrogen, they found the highest value for serine to be 17·7% greater than the lowest value, while for ethanolamine the corresponding figure was 32·6%. However, determinations on the same batch of reagent carried out a week apart were the same. Moore & Stein (1948) reported no such erratic behaviour and published a table of colour values for amino acids and other compounds based on leucine as 1·00 which has since been widely used.

Data on estimations using ninhydrin reagents are not usually recorded in absolute units, and although we have done so in the present paper it is probable that the molar colour yields obtained will vary appreciably with the purity of the reagents and the experimental conditions under which the estimations are made. It may, however, be noted that the colour yield per amino group of egg phospholipid (Fig. 1) is only 7% higher than that given by Fowden (1951) for alanine, although ethanolamine gives a weaker colour. The sensitivity of the method is excellent and reproducibility is adequate if reasonable precautions are taken.

The Van Slyke method. No explanation of the interference by unsaturated fatty acids with the Van Slyke estimation of amino N in intact phospholipids appears to have been given, although Folch *et al.* (1940) indicated (without quoting any other figures) that the magnitude of such interference was of the order of 0·12 mole nitrogen per unsaturated linkage, presumably for a reaction time of 10 min. at 23·6°. The present observations show a smaller effect (for a reaction time of 5 min. at 20°) which, however, increases with the number of double bonds present in the fatty-acid chain.

Labile methylene groups between two carbonyl groups are known to react with nitrous acid to give oximino compounds which, in turn, can react with nitrous acid to liberate nitrous oxide (Taylor & Baker, 1937) and it may be that in the case of the unsaturated fatty acids the reactive methylene groups adjacent to and particularly between the double bonds are involved. Whatever the precise nature of the side reactions with unsaturated fatty acids there seems little hope of applying the Van Slyke method to the quantitative determination of free amino groups in unhydrolysed phospholipids except in a very empirical and approximate way unless the unsaturated fatty acids are first destroyed, e.g. by hydrogenation.

SUMMARY

1. The ninhydrin procedure of Moore & Stein (1948), modified to maintain the lipids in solution, has been applied to the estimation of phosphatidylethanolamine (1–5 μg. N) in the presence of phosphatidylcholine (up to 1000 μg. N) without preliminary hydrolysis of the phospholipid.

2. Phosphatidylcholine as well as phosphatidylethanolamine liberates gas in the Van Slyke manometric procedure for the estimation of amino N owing to reaction of its component unsaturated fatty acids with nitrous acid. This method can therefore be applied only empirically to unhydrolysed phospholipids using a reaction time chosen for the particular material to agree with a determination on the fatty acid-free hydrolysate. It is much less sensitive than the ninhydrin method.

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Studies in Detoxication

58. THE METABOLISM OF ALIPHATIC ALCOHOLS. THE GLUCURONIC ACID CONJUGATION OF CHLORINATED AND SOME UNSATURATED ALCOHOLS

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Kamil, Smith & Williams (1953*a*) have shown that secondary and tertiary aliphatic alcohols are excreted by rabbits largely conjugated with glucuronic acid, and within the group of compounds studied, the extent of this conjugation increases with the number of carbon atoms. With primary alcohols, on the other hand, the conjugation is low. In general, the extent of conjugation increases in the order primary, secondary, tertiary. The reverse order appears to hold for the extent of oxidation of the alcohols *in vivo*. Although the pharmacological activity of alcohols depends on a number of factors, it does appear that, within an isomeric series, hypnotic activity increases as one passes from primary through secondary to tertiary alcohols (cf. Burger, 1951). In fact, the only simple alcohol which has found therapeutic use besides ethanol is tertiary pentanol (amylene hydrate). There thus appears to be a possible correlation between extent of glucuronic acid conjugation and hypnotic activity.

The introduction of halogens into organic compounds by substitution is often looked upon as a reasonable means of increasing pharmacological activity and, in the alcohol series, 2:2:2-trichloroethanol, 1:1:1-trichloro*iso*propanol (Isopral) and 1:1:1-trichloro-*tert.*-butanol (chloretone, chlorbutol) are much more powerful in their anaesthetic and hypnotic effects than the corresponding unsubstituted alcohols. It would therefore be expected that these substituted alcohols would be largely conjugated with glucuronic acid. The glucuronic acid conjugation of a number of chlorinated alcohols has therefore been measured and compared with that of the corresponding unsubstituted alcohols. Trichloroethanol is an active narcotic (Lehmann & Knoefel, 1938; 1939; Butler, 1948), and Butler (1949) has already shown that it is highly conjugated with glucuronic acid in the dog.

Primary alcohols are usually rapidly oxidized *in vivo* and their glucuronic acid conjugation is very low (Kamil *et al.* 1953*a*, *b*). Owing to the reported high conjugation of trichloroethanol, it was therefore of interest to study the effect upon glucuronic acid conjugation of progressive substitution of the β-carbon of ethanol with chlorine.

Recently the acetylenic tertiary alcohol, 3-methylpent-1-yn-3-ol (Oblivon, Dormison) has been recommended as a mild hypnotic, and it was of interest to include this in our studies. Acetylene itself was once used as a surgical anaesthetic, and Bock (1930) reported both diethylethynylcarbinol and *tert.*-butylethynylmethylcarbinol to produce sleep in dogs in doses of 0·2–0·25 g./kg. body wt. In order to assess the effect of the triple bond on the conjugation, the corresponding saturated alcohol, 3-methylpentan-3-ol (diethylmethylcarbinol) and the unsaturated 3-methylpent-1-en-3-ol were also studied. Propargyl alcohol (prop-2-yn-1-ol) was also fed but proved to be lethal to rabbits in doses of

about 0·1 ml./kg. body wt. in about 24 hr., thus confirming observations made by Tietze (1926) that this compound was toxic.

Finally diethylaminoethanol, which is itself a compound of low pharmacological activity, but occurs as a constituent of many useful drugs, such as procaine, was included. Brodie, Lief & Poet (1948) injected 1 g. of diethylaminoethanol into human beings and found about 33 % to be excreted unchanged in the urine in 24 hr.; the fate of the rest is unknown.

EXPERIMENTAL

Materials and methods

Materials. The following commercial products were purified by distillation: 2-chloroethanol (ethylene chlorohydrin), b.p. 129°; trichloro-*tert.*-butanol (chlorbutol), m.p. 91°; 1:3-dichloropropan-2-ol (α:γ-dichlorohydrin), b.p. 174–175°; diethylaminoethanol, b.p. 159°/753 mm. Dichloroethanol, b.p. 146°; trichloroethanol, b.p. 55°/20 mm., and 2:2:3-trichloro-*n*-butanol, m.p. 60°, were prepared by the reduction of dichloroacetic acid, trichloroacetaldehyde (chloral) and 2:2:3-trichlorobutyraldehyde, respectively, with $LiAlH_4$ according to the general method described by Brown (1951). 1:1:1-Trichloropropan-2-ol (Isopral), b.p. 160°, n_D^{20} 1·4821, was prepared from chloral and magnesium methyl iodide (cf. Bayer and Co., 1904); it solidified on keeping.

3-Methylpentan-3-ol (diethylmethylcarbinol), b.p. 122°, n_D^{20} 1·418, was purchased. 3-Methylpent-1-yn-3-ol (ethylethynylmethylcarbinol; Oblivon), b.p. 119°, n_D^{20} 1·430, was the gift of British Schering Ltd. 3-Methylpent-1-en-3-ol (ethylmethylvinylcarbinol), b.p. 117–118°, n_D^{20} 1·425 (cf. Zal'manovich, 1948) was prepared by reducing 3-methylpentyn-3-ol in methanol with platinic oxide (Adams's catalyst) and hydrogen.

Methods. Glucuronic acid in urine was determined by the naphthoresorcinol method as modified by Paul (1951).

Rabbits (weighing about 3 kg.) were maintained on a constant diet of 50 g./day of rat cubes (diet 41, Associated London Flour Millers).

Most compounds were administered in aqueous suspension by stomach tube, at a dose of 1·5 mmole/kg. body wt. Monochloroethanol could not be administered at this level because of its toxicity. Propargyl alcohol (prop-2-yn-1-ol) was fed to three rabbits at a dose level of 0·1 ml./kg. body wt. but all three animals died within 12 hr.; the urine excreted was highly reducing to Benedict's reagent but no glucuronide was excreted (see Table 1 for results).

Isolation of glucuronides (glucosiduronic acids)

The glucosiduronic acids were isolated from the urine as basic lead salts (cf. Kamil *et al.* 1951), using the urine remaining from the quantitative analyses. In three cases, including trichloroethanol, the free glucosiduronic acids were isolated. In the other cases the glucuronide gums were converted into crystalline triacetyl methyl esters (cf. Kamil *et al.* 1951). These esters were obtained as colourless needles, insoluble in water but soluble in ethanol and $CHCl_3$. With the di- and tri-chloroethanols, the yield of crystalline ester was more than 1 g./g. of alcohol fed; with the other alcohols the yield varied from 50 to 300 mg./g. Optical rotations, melting points and elementary analyses for these compounds are given in Table 2.

The molecular rotations of the triacetyl methyl esters prepared here are quoted in Table 2. The $[M]_D$ of compounds containing the $—CCl_3$ group are more negative than the corresponding unsubstituted derivatives.

The following free acids were prepared; 1:1:1-*trichloro*-tert.-*butyl*-β-D-*glucosiduronic acid*, m.p. 170–171° and $[\alpha]_D^{20}$ −37° in water (*c*, 1). (Found: C, 32·7; H, 4·5; Cl, 29·0. $C_{10}H_{15}O_7Cl_3$, H_2O requires C, 32·3; H, 4·6; Cl, 28·6 %); 2:2:3-*trichloro*-n-*butyl*-β-D-*glucosiduronic acid* (urobutylchloralic acid), m.p. 200–205° and $[\alpha]_D^{20}$ −40° in water (*c*, 1). (Found: C, 31·6; H, 5·3. $C_{10}H_{15}O_7Cl_3$, 1·5 H_2O requires C, 31·6; H, 4·8 %.) The K and Ag salts of the latter acid, a metabolite of butyl chloral hydrate, have been described by Külz (1882) and Mering (1882).

Table 1. *Glucuronic acid conjugation of some aliphatic alcohols in rabbits*

Alcohol	Formula	Dose (mg./kg.)	Dose (mmole/kg.)	Conjugation (% of dose)	Conjugation Average (%)
Ethanol*	CH_3CH_2OH	770	5	—	0·5
Monochloroethanol	CH_2ClCH_2OH	80	1	(Toxic)	0?
Dichloroethanol	$CHCl_2CH_2OH$	170	1·5	34, 65, 67	55
Trichloroethanol	CCl_3CH_2OH	220	1·5	49, 52, 62	52
*iso*Propanol*	$(CH_3)_2CHOH$	1000	17	—	10
1:3-Dichloro*iso*propanol	$(CH_2Cl)_2CHOH$	190	1·5	21, 25, 37	28
(±)-1:1:1-Trichloro*iso*propanol	$(CH_3)(CCl_3)CHOH$	240	1·5	31, 37, 39	36
Butanol*	$CH_3(CH_2)_2CH_2OH$	370	5	—	2
(±)2:2:3-Trichloro-*n*-butanol	$CH_3CHClCCl_2CH_2OH$	260	1·5	39, 43, 60	47
tert.-Butanol*	$(CH_3)_3COH$	296	4	—	24
1:1:1-Trichloro-*tert.*-butanol	$CCl_3(CH_3)_2COH$	260	1·5	33, 43, 49	41
Diethylaminoethanol	$(C_2H_5)_2NCH_2CH_2OH$	170	1·5	0, 0, 0	0
3-Methylpentan-3-ol	$(C_2H_5)_2COHCH_3$	150	1·5	39, 44, 60	48
(±)-3-Methylpenten-3-ol	$(C_2H_5)(CH_2{:}CH)COHCH_3$	150	1·5	52, 60, 60	57
(±)-3-Methylpentyn-3-ol	$(C_2H_5)(CH{:}C)COHCH_3$	150	1·5	43, 53, —	48

* These alcohols are included for comparison and the values quoted are taken from Kamil *et al.* (1953*a*, *b*).

Table 2. *The properties of the triacetyl methyl esters of certain biosynthetic glucuronides*

	Triacetyl methyl esters							
				Elementary analysis				
		Optical rotation in $CHCl_3$ (*c*, 1)		Found		Formula requires		
β-Glucuronide	M.p.*	$[\alpha]_D^{20}$†	$[M]_D$	C	H	C	H	Formula
Ethyl‡	143°	−32·8°	−119°	—	—	—	—	—
2:2-Dichloroethyl	163–164	−23	−99	41·9	4·75	41·8	4·7	$C_{15}H_{20}O_{10}Cl_2$
2:2:2-Trichloroethyl	158	−37	−172	38·9	4·2	38·7	4·1	$C_{15}H_{19}O_{10}Cl_3$
*iso*Propyl‡	140	−37·8	−132	—	—	—	—	—
1:3-Dichloro*iso*propyl	145–146	−27	−118	43·7	4·9	43·2	5·2	$C_{16}H_{22}O_{10}Cl_2$
1:1:1-Trichloro*iso*propyl§	167–169	−33	−158	40·2	4·6	40·1	4·4	$C_{16}H_{21}O_{10}Cl_3$
2:2:3-Trichloro-*n*-butyl§	132–133	−19	−94	41·5	4·8	41·4	4·7	$C_{17}H_{23}O_{10}Cl_3$
tert.-Butyl‡	168	−29·2	−114	—	—	—	—	—
1:1:1-Trichloro-*tert.*-butyl	170	−28	−138	42·0	4·5	41·4	4·7	$C_{17}H_{23}O_{10}Cl_3$
3-Methylpentyl-3-ol	111	−24	−100	54·4	7·0	54·5	7·2	$C_{19}H_{30}O_{10}$
3-Methylpentenyl-3-ol§	99–105	−19	−79	55·0	6·9	54·8	6·8	$C_{19}H_{28}O_{10}$
3-Methylpentynyl-3-ol§	132–134	−14	−58	55·1	6·2	55·1	6·3	$C_{19}H_{26}O_{10}$

* Melting points are uncorrected.
† The error in $[\alpha]_D$ is ±1°.
‡ Included for comparison of rotations (values quoted from Kamil *et al.* 1953*a*, *b*).
§ The (±) alcohols were fed; the glucuronides are assumed to contain the aglycone in the (±) form.

DISCUSSION

The glucuronic acid conjugations of the alcohols studied are given in Table 1. It is to be noted that in all cases, except monochlorethanol, the introduction of chlorine atoms into the molecule causes a large increase in the glucuronic acid conjugation. Thus the oxidation of the alcohols appears to be inhibited by the presence of chlorine atoms. Whereas ethanol is largely oxidized to acetic acid, more than a half of the di- and trichloroethanols are excreted as glucuronides. However, no extra glucuronic acid excretion was detected in the urine during 12 hr. after dosing with monochloroethanol, and it seems probable that it is readily oxidized to monochloroacetic acid (the monochloroethanol was fed at a dose level of 80 mg./kg. body wt., but the animals died in 12 hr. after dosing; the LD_{50} for rats is 72 mg./kg. (Goldblatt, 1944; Goldblatt & Chiesman, 1944)). Monochloroacetic acid is a fairly toxic substance, being 25–40 times more toxic to rats, mice and guinea pigs than acetic acid and its di- and trichloro derivatives (Woodard, Lange, Nelson & Calvery, 1941). This acid also markedly depresses the respiration of mouse-liver slices under conditions where acetic acid and di- and tri-chloroacetic acids have little effect (Laug, 1946). It is reasonable to suppose that the toxicity of monochloroethanol is due to the production of monochloroacetic acid (cf. fluoroethanol and fluoroacetic acid; Reilly, Riker, Whitehouse & Kuriaki (1953) have suggested that the toxic action of monochloroacetate like that of monofluoroacetate may be due to an inhibition of pyruvate metabolism, although citrate accumulation does not occur with monochloroacetate). Thus it appears that glucuronic acid conjugation only becomes a significant metabolic path when two chlorine atoms have been substituted into the β-carbon of ethanol, and that di- and trichloroethanol are equally well conjugated. Butler (1948) has already shown that the oxidation of trichloroethanol to trichloroacetic acid in dogs is insignificant, the main metabolite being trichloroethyl glucosiduronic acid.

A similar effect is to be noted with 2:2:3-trichloro-*n*-butanol. Butanol itself is readily oxidized and has a low glucuronic acid conjugation in the rabbit (Kamil *et al.* 1953*a*), but in the 2:2:3-trichloro derivative two chlorine atoms occur on the β-carbon atom and this compound becomes highly conjugated with glucuronic acid.

The effect of chlorine substitution on the metabolism of a secondary alcohol is shown in the case of *iso*propanol. 1:2-Dichloro and 1:1:1-trichloro-*iso*propanol in low doses have much higher conjugations than *iso*propanol in high doses. Here again it appears that the oxidation to the ketone has been retarded. A similar effect is to be noted with *tert.*-butanol and its trichloro derivative.

Of the above chlorinated alcohols, trichloroethanol, 1:1:1-trichloro*iso*propanol (Isopral) and 1:1:1-trichloro-*tert.*-butanol (Chlorbutol) have found use as hypnotics, and it is possible that butyl chloral hydrate may be active because of reduction *in vivo* to 2:2:3-trichloro-*n*-butanol (cf. chloral hydrate). These four alcohols are excreted by rabbits highly conjugated with glucuronic acid.

It thus appears that chlorine substitution tends to retard the oxidation of alcohols and this may partly explain why the chlorinated alcohol has a

more powerful and more prolonged narcotic effect than the unsubstituted compound. However, conjugation with glucuronic acid is also an inactivating reaction, but it is localized mainly in the liver. It is also possible that chlorine substitution also retards the rate of glucuronic acid conjugation, so that the narcotic effectiveness of the substituted alcohol may be partly due to inhibition of oxidation and retardation of conjugation.

At the end of Table 1, values for the glucuronic acid conjugation of the three related tertiary alcohols, 3-methylpentan-3-ol, 3-methylpent-1-en-3-ol and 3-methylpent-1-yn-3-ol, are included to show the effect of unsaturation upon conjugation. The total conjugations of these three alcohols are not very different and it appears that the conversion of ethyl into vinyl or to ethynyl has little effect on conjugation. 3-Methylpentyn-3-ol (Oblivon, Dormison, Methylparafynol) has mild narcotic properties but nothing appears to be known about the narcotic properties of the other two alcohols.

SUMMARY

1. The glucuronic acid conjugation of a number of chlorinated and other aliphatic alcohols has been studied in the rabbit, and the glucosiduronic acids have been isolated as triacetyl methyl esters in each case.

2. Conjugation with glucuronic acid becomes a significant metabolic path when two or more chlorine atoms are present on the carbon atom next to that carrying the hydroxyl group. Thus 2-chloroethanol is not conjugated, whereas 2:2-di- and 2:2:2-tri-chloroethanols are highly conjugated (more than 50%).

3. It is suggested that chlorine substitution retards the oxidation of the alcohol and thereby increases its narcotic activity.

4. 3-Methylpentan-3-ol, 3-methylpent-1-en-3-ol and 3-methylpent-1-yn-3-ol are conjugated with glucuronic acid to about the same extent (50%). Diethylaminoethanol does not cause the excretion of extra glucuronic acid in rabbits.

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A Note on the Estimation of Sphingomyelin in Nervous Tissue

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The estimation of sphingomyelin in animal tissues is almost exclusively carried out by measuring the lipid phosphorus which is stable to mild alkaline and acid hydrolysis. Apart from sphingomyelin, all the known phospholipids are decomposed by incubating with N potassium hydroxide at 37° followed by standing at room temperature at an acid pH (Schmidt, Benotti, Hershman & Thannhauser, 1946). However, the work of Brante (1949) has suggested that certain kephalin fractions isolated from cerebral tissue contain some lipid phosphorus which is stable to mild alkaline and acid hydrolysis, although the fractions themselves contain no choline.

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It was found in the present experiments that when sphingomyelin was estimated in brain tissue by the method of Schmidt *et al.* (1946) lower values were obtained when the lipids in the tissue were initially precipitated with trichloroacetic acid (TCA) before they were extracted with solvents. This has been shown to be due to a loss during the precipitation of non-sphingomyelin lipid phosphorus which becomes insoluble in TCA after alkaline hydrolysis and consequently is estimated as sphingomyelin. Even after the precipitation of brain lipids with TCA, the molar choline/phosphorus ratio of the alkali-stable 'sphingomyelin' is less than unity, indicating that the method of Schmidt *et al.* (1946) is unsuitable for the accurate estimation of this phospholipid.

METHODS

Extraction procedure. Brain tissue was removed from adult guinea pigs soon after death and kept frozen at −20° until required. It was then finely minced and two representative samples were weighed. One sample was ground in a mortar with 5 or 10% (w/v) TCA solution, the precipitate centrifuged off, and washed once with water. The lipids were then extracted from the precipitate and the untreated sample by various solvent-extraction procedures; the initial extraction being always carried out with cold solvents to eliminate the possibility of residual TCA causing phospholipid hydrolysis. The combined extracts of the two samples were each filtered and made up to 500 ml. Occasionally a fine precipitate separated from the direct extract, and was removed. For the estimation of 'alkali-stable' lipid P duplicate samples were taken to dryness *in vacuo* at room temp. and then either directly, or after a further re-extraction with solvents, the lipid residue was incubated for 18–24 hr. with N-NaOH or KOH at 37°. The hydrolysates were cooled in ice, and neutralized with 6N-HCl. An equal volume of 10% (w/v) TCA was then added and after standing 2 hr. at room temp., the precipitated 'alkali-stable' phospholipids were filtered off and copiously washed on the filter paper with large quantities of 5% (w/v) TCA. The paper and the lipids were then completely digested and assayed for phosphorus.

Estimations. Phosphorus was determined by the Fiske & Subbarow method (1925). Choline estimations were performed in duplicate by the procedure of Entenman, Taurog & Chaikoff (1944) after the 'alkali-stable' phospholipids had been filtered off through an asbestos pad and hydrolysed with baryta. If any of the precipitates of choline reineckate was amorphous, all the precipitates, including those of the standards, were crystallized by the procedure of Schmidt, Hecht, Fallot, Greenbaum & Thannhauser (1952). The term 'alkali-stable' P used throughout this paper denotes, for convenience, that phosphorus which remains insoluble after incubation with N-KOH at 37° (24 hr.) followed by standing in 5% TCA for 2 hr. at room temp.

RESULTS

When brain tissue is precipitated with TCA at room temperature before the lipids are extracted with solvents, the 'alkali-stable' lipid P found in the extracts is appreciably lower than that recovered from a direct extract of the tissue (Table 1). This apparent lowering of the sphingomyelin content of the lipid extract was observed with three different procedures for lipid extraction, and also when magnesium chloride was added to the TCA solution to improve the precipitation of the lipid material (Johnson & Dutch, 1951). The decrease appeared to be no greater when dissected white matter was used rather than whole brain tissue. Similar, if somewhat smaller, decreases were observed with samples of lung and liver tissue. The decrease in 'alkali-stable' lipid P accounted for a considerable part of the loss of total lipid P found after the TCA precipitation of brain tissue (Table 1). This loss of total lipid P after TCA precipitation has been observed in other tissues (e.g. Swanson & Artom, 1950), and was

Table 1. *The recovery of total and 'alkali-stable' lipid* P *from guinea pig tissues by direct lipid extraction and by extraction of lipids after TCA precipitation*

Results are given as mg. P/100 g. fresh tissue.

Tissue	Method of lipid extraction	Lipid precipitant	Total lipid P		'Alkali-stable' lipid P		Recovery of 'alkali-stable' lipid P after TCA precipitation (%)
			Direct extraction	Extracted after TCA precipitation	Direct extraction	Extracted after TCA precipitation	
Brain	Johnson, McNabb & Rossiter (1948)	10% (w/v) TCA	249 188	195 176	34·9 28·0	20·4 18·6	58 66
Brain	McKibbin & Taylor (1949)	10% (w/v) TCA + 0·4M-$MgCl_2$	—	—	30·6	22·0	72
Brain	Schmidt, Benotti, Hershman & Thannhauser (1946)	10% (w/v) TCA + 0·4M-$MgCl_2$	224	209	30·6	21·6	71
Brain		10% (w/v) TCA	245	214	32·7	24·8	76
Brain		5% (w/v) TCA	228	208	33·5	22·8	68
Brain white matter		10% (w/v) TCA	286	266	49·1	36·5	74
Lung		10% (w/v) TCA + 0·4M-$MgCl_2$	104	98	15·7	13·2	84
Liver			150	131	14·8	12·9	87

generally considered to be due to the removal of acid-soluble phosphorus which contaminates lipid extracts (McKibbin & Taylor, 1949), and also the partial hydrolysis of plasmalogens (Feulgen & Bersin, 1939).

It was clear that the lower recovery of 'alkali-stable' lipid phosphorus found after TCA precipitation was caused either by the precipitant rendering some of this phosphorus non-extractable by lipid solvents or by its removal in the acid-soluble fraction. The former alternative was tested by measuring the 'alkali-stable' P both in the lipid extract and also in the residual P fraction obtained after the solvent extraction of the lipids (Table 2). The 'alkali-stable' P found in this residual fraction was slightly higher when the tissues were precipitated with TCA prior to solvent extraction of the lipids. However, it is apparent from Table 2 that this small increase in 'alkali-stable' residual P could not nearly account for the loss of 'alkali-stable' P from the lipid extract. When measurements were made of the total 'alkali-stable' P present in brain tissue before and after TCA precipitation, it was found that the precipitation resulted in a considerable loss of phosphorus (Table 3). When the precipitation of the total 'alkali-stable' P was carried out at 0°, a similar, although smaller, loss of phosphorus occurred. If, therefore, it is assumed that the cerebral deoxyribonucleic acid P is completely recoverable in the TCA precipitate (Schmidt & Thannhauser, 1945) this result would suggest that most of the 'alkali-stable' lipid P lost during the precipitation becomes soluble in the precipitant.

Table 2. *Estimation of 'alkali-stable' lipid and residual* P *in guinea pig brain after direct lipid extraction and extraction of lipids after TCA precipitation*

Results are given as mg. P/100 g. fresh tissue.

		'Alkali-stable' lipid P		'Alkali-stable' residual P	
Tissue	Lipid precipitant	Direct extraction	Lipids extracted after TCA precipitation	Direct extraction	Lipids extracted after TCA precipitation
Brain	7% (w/v) TCA	38·4	24·0	7·7	11·3
	10% (w/v) TCA	34·6	23·5	7·8	10·4
Brain white matter	10% (w/v) TCA	—	—	6·0	7·4
	10% (w/v) TCA	49·1	36·5	7·1	8·2

Table 3. *The loss of total 'alkali-stable'* P *during precipitation of guinea pig brain tissue with TCA*

Expt. no.	Conditions of precipitation	Total 'alkali-stable' P (mg. P/100 g. fresh tissue)	'Alkali-stable' P lost by precipitation (mg. P/100 g. fresh tissue)
1	No precipitation, direct lipid extraction	49·4	—
	Precipitation with 10% (w/v) TCA at room temp.	37·8	11·6
2	No precipitation, direct lipid extraction	48·1	—
	Precipitation with 10% (w/v) TCA at room temp.	34·0	14·1
	Precipitation with 10% (w/v) TCA at 0°	39·1	9·0
3	No precipitation, direct lipid extraction	52·1	—
	Precipitation with 5% (w/v) TCA at room temp.	38·9	13·2
	Precipitation with 5% (w/v) TCA at 0°	49·5	2·6

Table 4. *The effect of TCA precipitation on the choline content of the 'alkali-stable' lipids of brain*

Expt. no.	Method of preparation of 'alkali-stable' lipids	Choline (mg./100 g. fresh tissue)	Molar ratio choline/ phosphorus
1	Direct lipid extraction	70	0·69
	Extraction of lipids after precipitation of brain with 5% (w/v) TCA (room temp.)	69	0·91
	Extraction of lipids after precipitation of brain with 5% (w/v) TCA (0°)	68	0·76
2	Direct lipid extraction	78	0·61
	Extraction of lipids after precipitation of brain with 5% (w/v) TCA (room temp.)	81	0·89
3	Direct lipid extraction	39	0·39
	Extraction of lipids after precipitation of brain with 10% (w/v) TCA (room temp.)	44	0·58

It therefore became important to know whether the loss of 'alkali-stable' lipid P occurring during TCA precipitation of the tissue was associated with an equivalent loss of choline. It was found, however, that the precipitation resulted in no appreciable loss of choline from the 'alkali-stable' phospholipids (Table 4). When the lipids were directly extracted from the tissue the 'alkali-stable' lipids had a molar choline/phosphorus ratio which was considerably less than unity. Although the loss of 'alkali-stable' lipid P during TCA precipitation raised this ratio somewhat, it was still less than unity, suggesting that the 'alkali-stable' phospholipids still did not consist entirely of sphingomyelins.

DISCUSSION

The finding that the molar choline/phosphorus ratio of the 'alkali-stable' brain lipids is less than unity suggests that other phospholipids apart from sphingomyelins are present in this fraction. The estimation of choline by reineckate precipitation is notoriously affected by interfering substances and therefore, though good recoveries of added choline were obtained from hydrolysates of the fraction, such evidence on its own cannot be regarded as conclusive. However, the observation that TCA precipitation of brain tissue removes considerable amounts of lipid P from the subsequently isolated 'alkali-stable' lipid fraction without removing choline, confirms that this fraction, isolated from direct lipid extracts, cannot consist entirely of sphingomyelin. The results suggest that this conclusion may also apply to other tissues, and in this connexion Mallov, McKibbin & Robb (1953) have recently reported that in fresh lipid extracts of heart muscle, the sphingomyelin P measured by the procedure of Schmidt *et al.* (1946) was much greater than could be accounted for by the sphingosine present in the extract.

The identity of this 'alkali-stable' lipid phosphorus which is not sphingomyelin is not clear. None of the known phospholipids existing in a free state are stable to mild alkaline and acid hydrolysis, except sphingomyelin. The possibility must be considered that the phosphorus is contained in some compound which is not a lipid as the only evidence indicating it is combined in a lipid is its solubility in fat-solvents, and its insolubility in TCA solution. It seems more likely, however, that the phosphorus is derived from a new phospholipid or from some known phospholipid present in a modified or combined form. Present attempts to identify any base in the 'alkali-stable' phospholipids, apart from choline and sphingosine, have been foiled by the presence of protein material derived from proteolipids in the fraction. No more than traces of glycerophosphoric acid could be found in total hydrolysates of the 'alkali-stable' lipids. It is not likely that the substance is produced by a rapid post-mortem enzymic action as a similar effect of TCA precipitation has been observed with rat brain tissue frozen *in situ* with liquid oxygen.

The solubility of the non-sphingomyelin 'alkali-stable' lipid phosphorus during an initial TCA precipitation of the tissue is somewhat puzzling, as after alkaline incubation of the directly extracted lipids it estimates as 'alkali-stable' lipid P, even though it stands for a considerably longer period in contact with TCA solution. The stability of the plasmalogens seems to be the reverse of this behaviour; in crude lipid extracts they are relatively stable to acid conditions, while after alkaline incubation or in the free state they are quickly decomposed (Schmidt *et al.* 1946; Schmidt, Ottenstein & Bessman, 1953; Ansell & Norman, 1953). The possibility of incomplete precipitation by the TCA is unlikely as the resulting TCA solution contains only a negligible quantity of phosphorus which estimates as sphingomyelin.

The estimation of sphingomyelin P in nervous tissue by alkaline hydrolysis therefore only gives an approximate result. Although the accuracy can be improved somewhat by initially precipitating the tissue with TCA, it is probable that for a more precise determination it is preferable to estimate the choline in the 'alkali-stable' lipids or to measure the difference between lecithin and total choline (Brante, 1949).

SUMMARY

1. The molar choline/phosphorus ratio of the cerebral lipids which are stable to mild alkaline and acid hydrolysis is less than unity, indicating that sphingomyelins are not the only phospholipids present.

2. Part of this non-sphingomyelin lipid phosphorus is removed by initially precipitating the tissue lipids with trichloroacetic acid at room temperature, a procedure which does not affect the recovery of sphingomyelin.

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Some Enzymic Changes in the Mammary Gland of Rats during Pregnancy, Lactation and Mammary Involution

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Recent work has emphasized that the lactating mammary gland is a site of intense synthetic activity (see Folley, 1949). During the past five years, fat synthesis in the gland has received considerable attention, but protein synthesis has been relatively neglected, although the mammary gland is known to synthesize large quantities of specific proteins, and, since these are promptly secreted into the milk, an equilibrium condition is never reached and the high rate of protein synthesis is maintained.

The application of isotopic methods to the study of milk-protein precursors by Campbell & Work (1952) is clearly a most promising line of investigation, but it seemed to us that the study of some of the enzymes involved might also yield interesting information. In view of the present state of knowledge of the mechanism of protein synthesis, it seemed more profitable to make a general survey of some of the enzymes which might be implicated in these processes, rather than an intensive study of a particular enzyme system. We have therefore studied glutamic dehydrogenase, glutamic–aspartic transaminase and cathepsin, which have been suggested as playing some part in protein synthesis. In addition, we have measured the activity of β-glucuronidase, first because this enzyme is also found in the particulate fractions (Walker & Levvy, 1953) and is one which, because it is believed not to be concerned with tissue growth in the rat, would differentiate between a specific enzymic change and a general change in the level of mitochondrial enzymes; and secondly because of its postulated role in oestrogen metabolism (Fishman, 1947).

EXPERIMENTAL

Animals. Virgin rats of the hooded Norway strain of the Medical Research Council, aged 3 months, were mated with males of the same strain and age. Nine groups of rats, each containing five animals, were used; three groups of pregnant animals which were killed on the 10th, 15th and 20th days of pregnancy, four groups which were killed on the 5th, 10th, 15th and 20th days of lactation and two further groups which had their litters removed on the 21st day of lactation and were killed on the 2nd and 4th days of mammary involution. During lactation the litter size was reduced to eight pups, four male and four female, where possible. Rats were killed by dislocation of the cervical vertebrae and the three abdominal mammary glands quickly removed, weighed and disintegrated in water in a Folley & Watson (1948) blender to give a final tissue concentration of 1:5 for glutamic dehydrogenase and transaminase estimations, and 1:10 for cathepsin and β-glucuronidase estimations.

Materials. α-Oxoglutarate was prepared by the method of O'Kane (1949) and was 96% pure by carbonyl-group estimation.

Cytochrome *c* was prepared by the method of Keilin & Hartree (1945) from horse heart. This had a concentration of $3{\cdot}26 \times 10^{-3}$ M as estimated by the method of Potter (1941).

Diphosphopyridine nucleotide (DPN) was prepared by the method of Williamson & Green (1940); various preparations gave assays between 55 and 65% pure.

p-Chlorophenyl glucosiduronic acid was prepared by the method of Spencer & Williams (1951). We found that a better yield could be obtained if the dose of *p*-chlorophenol (0·5 g./kg. of rabbit in 50 ml. of water) was divided into two portions and one given at 10 a.m. and the other at 6 p.m.

Haemoglobin was prepared by the method of Anson (1937) and stored in the freeze-dried state. For use, a 5% (w/v) solution of haemoglobin in water was prepared fresh each day.

Chemical methods. Lactose was estimated by the method of Hinton & Macara (1927) as modified by Folley & Greenbaum (1947). The milk content of the mammary gland suspensions was calculated from the lactose content and all enzyme assays were corrected for the 'milk error' as described by Folley & Greenbaum (1947).

Enzymic methods. Glutamic dehydrogenase was measured by the method described by Copenhaver, McShan & Meyer (1950), except that the reaction was carried out in the presence of 0·1 M nicotinamide, in view of the observation of Moore & Nelson (1952) that mammary gland contains an active nucleotidase and that of Terner (1952), who also

found a rapid destruction of DPN, and the protection of DPN by added nicotinamide. The results of this determination are expressed as μl. O_2 uptake/hr./g. wet wt. mammary tissue corrected for the presence of milk (Folley & Greenbaum, 1947).

Transaminase was measured at three tissue levels by the method of Greenwood & Greenbaum (1953). Protein precipitation from mammary-gland suspensions leaves a pad of fatty material floating on the surface after centrifugation and the removal of this was best effected by filtration. The results are expressed as μmoles oxaloacetic acid produced/hr./g. wet wt. of mammary gland corrected for the presence of milk.

Cathepsin. The method described by Anson (1938) was used, except that the scale of operations was reduced by a factor of 4. The concentration of haemoglobin solution was also double that used by Anson (1938) since, at this concentration, a linear relationship between the level of the enzyme and the tyrosine produced over the range 5–25 mg. of tissue (Fig. 1) was obtained. No activation of catheptic activity by cysteine, as suggested by Anson (1937), could be detected and accordingly no cysteine was added to the reaction mixture. Laskowski & Laskowski (1951) have demonstrated the presence of a powerful anti-trypsin in bovine colostrum. We have tested for the presence of such an inhibitor in suspensions of mammary gland taken from rats on the 20th day of pregnancy by assaying separately the catheptic activity of three mammary-gland suspensions, one at the 20th day of pregnancy, one at the 10th day of lactation and a mixture of equal volumes of these two suspensions. The value for the mixture was exactly the arithmetical mean of the two separate values, so that any inhibitor present had no action on the cathepsin assay used. Throughout these determinations the same batch of haemoglobin was used to ensure uniformity of substrate.

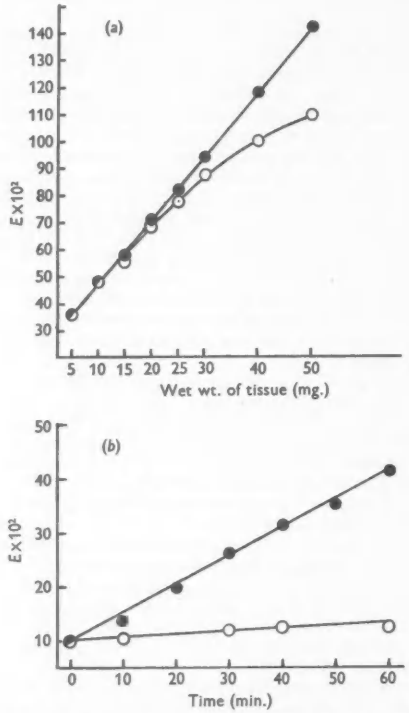

Fig. 1. (*a*) Relation between cathepsin concentration and reaction velocity in rat mammary-gland tissue suspensions, with substrate concentrations of 5% (w/v) haemoglobin (●) and 2·5% (w/v) haemoglobin (○). All experiments run for 15 min. at 37° and at pH 3·7. (*b*) Cathepsin reaction-velocity curves for rat mammary-gland tissue suspensions. All experiments using 10 mg. wet wt. of tissue; substrate concentration 5% (w/v) haemoglobin; temperature 37°; pH 3·7 (●). The open circles represent the incubated controls.

Preliminary experiments using this method have established that the liberation of tyrosine is linear over a period of 1 hr. (Fig. 1) and a 15 min. incubation period was chosen for routine use. Since the presence of a cathepsin in mammary gland had not previously been described in the literature, a pH characterization of the enzyme was carried out (Fig. 2). A sharp optimum was found at pH 3·7 and the estimation was therefore routinely carried out at that pH. The 1:10 tissue suspension was diluted with an equal volume of 0·2M acetate buffer, pH 3·7, immediately before use. Each determination was carried out at three tissue levels, 5, 15 and 25 mg. uncorrected wet wt., and the reaction product measured colorimetrically, using an Ilford Spectrum Red filter no. 608, transmitting from 6200 Å onwards, and a standard tyrosine curve. The results are expressed as mg. tyrosine/hr./g. of wet wt. of mammary tissue corrected for the presence of milk.

β-Glucuronidase was measured by the method of Spencer & Williams (1951). Owing to the high fat content of the mammary gland, a cloudy solution was obtained when the supernatant was diluted according to the above method. By diluting with freshly prepared 0·2N-KOH in 80% (v/v) ethanol instead of the aqueous 0·2N-NaOH recommended,

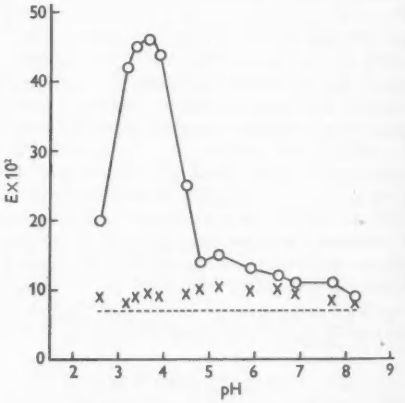

Fig. 2. pH-optimum curve of rat mammary-gland cathepsin (○). '- - - -' Represents the reagent blank and '×' represents the values for the incubated controls.

the final solution was found to be suitable for spectrophotometric measurements for periods up to 1 hr. For β-glucuronidase measurements, the 1:10 tissue suspension was diluted with an equal volume of 0·2 M acetate buffer, pH 5·2. Each determination was performed at three tissue levels, 5, 12·5 and 20 mg. uncorrected wet wt. A straight-line relationship exists between the increase in optical density due to the liberated *p*-chlorophenol and the tissue level up to 40 mg. of tissue. Walker & Levvy (1951, 1953) have suggested that the maximum activity of β-glucuronidase in rat-liver suspensions can only be realized in the presence of a non-ionic detergent which serves either to render the enzyme from the particles soluble or to overcome the inhibition due to some unknown endogenous inhibitor. We have attempted to assay β-glucuronidase in mammary-gland tissue suspensions using the detergent used by Walker & Levvy, Triton-X-100, in the concentrations they suggest, but we have found that the effect of this substance is to produce cloudy filtrates owing to the presence of stabilized fat droplets. Such filtrates were also found after the precipitation of the protein either by the method of Spencer & Williams (1951) or of Talalay, Fishman & Huggins (1946). It was impossible, therefore, to evaluate the effect of Triton-X-100 in the mammary gland.

However, Walker & Levvy (1953) have found that the use of citrate buffer in place of acetate also overcomes the inhibition and, in fact, gives values closely approximating to those obtained using Triton-X-100. We have made several determinations of mammary-gland β-glucuronidase in the presence of both these buffers and find no difference in the values obtained. It would appear, therefore, that rat mammary gland differs in this respect from rat liver.

RESULTS

Changes in the levels of the various enzymes measured during the lactation cycle are shown in Figs. 3–6.

Glutamic dehydrogenase. No change in the activity of this enzyme is detectable between the 10th and 15th day of pregnancy, but a highly significant increase ($P < 0{\cdot}001$) occurs between the 15th and 20th days (Fig. 3). This rise probably occurs abruptly later than the 17th day, since we have several results, not included in Fig. 3, at 16 and 17 days of pregnancy, all of which clearly fall in the same grouping as those at the 15th day.

By the 5th day of lactation the level had declined ($P < 0{\cdot}001$) but thereafter increased again to a maximum at 15 days of lactation, which is more or less maintained until the end of lactation. Very little change is noted in the first 2 days of mammary involution, but between the 2nd day and the 4th day the level drops precipitously.

The curve showing the total glutamic dehydrogenase activity in the three abdominal mammary

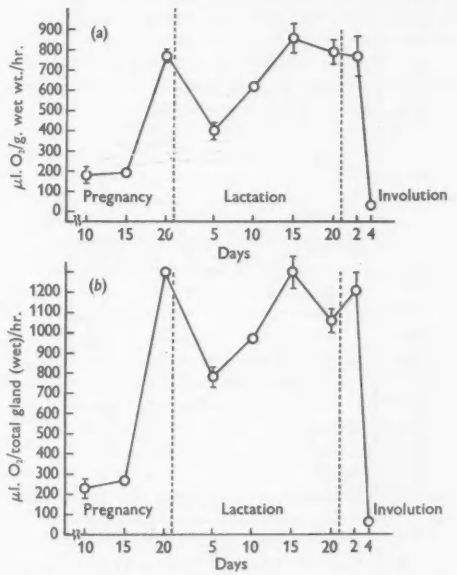

Fig. 3. Changes in the glutamic dehydrogenase activity of rat mammary-gland tissue suspensions: (*a*) activity/g. moist tissue, (*b*) total activity in three abdominal glands. Each point represents the mean of five animals. The vertical lines represent twice the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

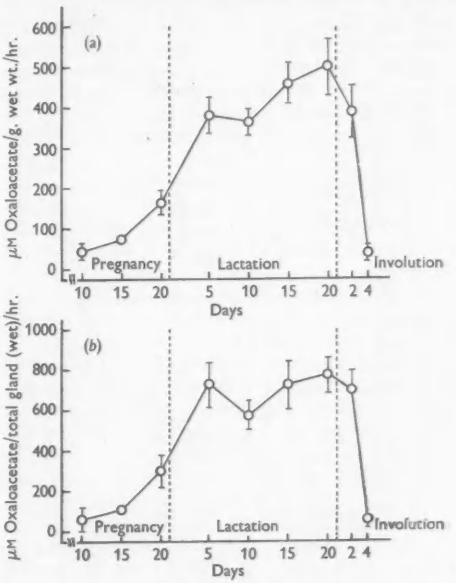

Fig. 4. Changes in the glutamic–aspartic transaminase activity of rat mammary-gland tissue suspensions: (*a*) activity/g. moist tissue, (*b*) total activity in three abdominal glands. Each point represents the mean of five animals. The vertical lines represent twice the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

glands follows a very similar course. The level of activity in this tissue is of a low order, being about one-tenth of that in liver.

Transaminase. The level of this enzyme (Fig. 4) increases slowly during pregnancy and rises abruptly at parturition so that at the 5th day of lactation the level is over double that found at the end of pregnancy ($P = 0{\cdot}006$). This high level is maintained, and even increases slightly, throughout the remainder of lactation. There is a slight decline in the first 2 days of mammary involution but in the next 2 days the level falls virtually to zero. The curves showing the level of enzyme/g. of tissue and the total enzyme are of similar form.

The activity of the mammary enzyme is about one-third of that found in liver.

Cathepsin. The changes in the level of cathepsin are shown in Fig. 5. Very little change is observed in the level of cathepsin/g. of tissue over the second half of pregnancy, but after parturition there is a steady increase, so that by the 20th day of lactation the level is over four times that obtaining on the 20th day of pregnancy ($P < 0{\cdot}001$). This rise continues into the period of mammary involution. The curve for the total cathepsin activity follows essentially the same course, except that it reaches a well-marked peak on the 2nd day after weaning and is still high on the 4th day, indicating that the increase in activity/g. from the 2nd to the 4th days of involution takes place less rapidly than the loss in dry weight of the gland over the same period.

The catheptic activity found in the mammary gland greatly exceeds that of other tissues examined, the value at the 10th day of lactation being over double that for lymph nodes, the most active tissue so far recorded (Rothschild & Junqueira, 1951).

β-Glucuronidase. Changes in the β-glucuronidase activity are shown in Fig. 6. The activity of the enzyme/g. of tissue increases but slightly between the 10th and 15th days of pregnancy ($P = 0{\cdot}50$), but rises to a sharp maximum by the 20th day ($P = 0{\cdot}001$). The level then declines progressively throughout the course of lactation, although it does not fall as low as that found up to the 15th day of pregnancy. There is a dramatic increase in the level

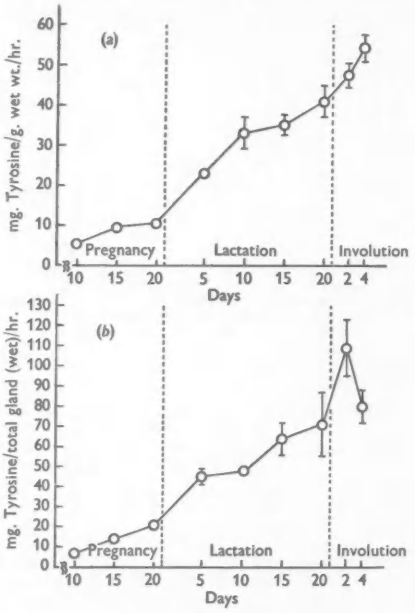

Fig. 5. Changes in the cathepsin activity of rat mammary-gland tissue suspensions: (*a*) activity/g. moist tissue, (*b*) total activity in three abdominal glands. Each point represents the mean of five animals. The vertical lines represent twice the S.E.M. Where no line is given the S.E.M. is too small to show.

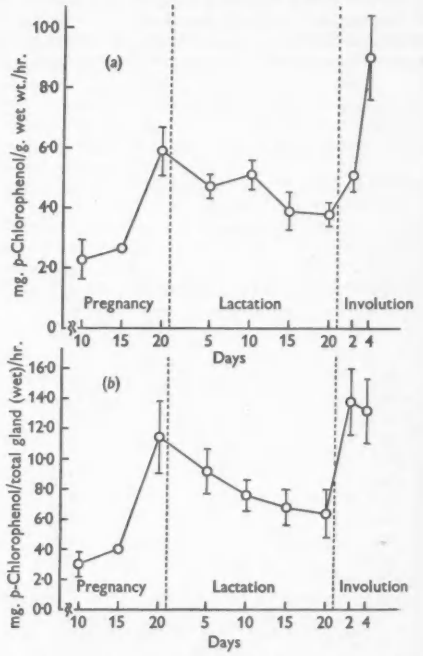

Fig. 6. Changes in the β-glucuronidase activity of rat mammary-gland tissue suspensions: (*a*) activity/g. moist tissue, (*b*) total activity in three abdominal glands. Each point represents the mean of five animals. The vertical lines represent twice the S.E.M. Where no vertical line is given the S.E.M. is too small to show.

of the enzyme during involution and indeed the highest value recorded, at the 4th day of mammary involution, is over twice the value found at the end of lactation ($P = 0{\cdot}007$). A similar trend is seen in the total β-glucuronidase activity, although here the peak value is found on the 2nd day of involution, the 4th day value being greatly decreased by the fall in weight of the regressing gland.

The β-glucuronidase activity of mammary gland is approximately one-third of that found in rat liver by Mills, Paul & Smith (1953) and Walker & Levvy (1953) using a different method of assay. For the purpose of this comparison we have recalculated our data into the units used by these authors.

DISCUSSION

The original suggestion of Braunstein & Azarkh (1945) that the coupling of the transaminating systems with glutamic dehydrogenase could play an important part in both the degradation and synthesis of amino acids has gained in significance from the demonstration by Cammarata & Cohen (1950) of the wide scope of the transamination reaction. The mechanism of urea synthesis put forward by Ratner (1949) proposes a scheme for amino acid catabolism which also centres round the interaction of these two systems. Since all the reactions in the Ratner scheme are reversible, the same scheme could be interpreted as a possible route for amino acid synthesis. A great deal of attention has been devoted to establishing a relationship between transaminase activity and protein synthesis. The fall in transaminating activity found by Cohen & Hekhuis (1941) in growing tissues led these authors to postulate an inverse relationship between protein synthesis and transamination activity, a conclusion also reached by Bartlett & Glynn (1950*a*) as a result of their study of muscle transaminase after hypophysectomy. On the other hand, Bartlett & Glynn (1950*b*) have found an increased transaminase activity in the livers of hypophysectomized rats treated with growth hormone and suggest that the increase was due to an increased synthesis of amino acids required for protein deposition.

The contrary nature of these results is not surprising, since a high level of transaminating activity could equally well indicate an increased level of amino-acid synthesis or an increased rate of degradation. According to the scheme indicated earlier, the arbiter of whether an amino group, when once transferred to α-oxoglutarate to form glutamate, is then again transferred to another keto acid to form a new amino acid, or is split off and passed into the ornithine cycle, would be the functional level of glutamic dehydrogenase.

In view of these considerations one might expect to find an inverse relationship between transaminase and glutamic dehydrogenase in the protein-synthetic phase of the lactation cycle, while in conditions where amino acid degradation predominates both enzymes should change in the same direction. Lactation in the rat is characterized by a progressive rise in milk yield (Brody & Nisbet, 1938) and hence of protein output. That the inverse relationship of the two enzymes does not hold in lactation can be seen from Figs. 3 and 4. If these results can be interpreted in terms of the Ratner scheme, then the changes of glutamic dehydrogenase and transaminase appear rather to indicate an increased amino acid degradation, a conclusion which it is difficult to reconcile with the known synthetic activity of the gland. However, a consideration of the changes in the morphological structure of the gland could lead to a different interpretation of these results.

Weatherford (1929) has reported a progressive increase in the number of mitochondria in the secretory cells of the mammary gland during pregnancy and lactation and a change in shape from a globular to a filamentous form. Jeffers (1935) has confirmed the change in form, and Dempsey, Bunting & Wislocki (1947) the increased number, of mitochondria through pregnancy and lactation. If such an increase really does occur then it is probable that the enzymes located in the mitochondria would increase at the same rate in a non-specific way. This could account for the rise in glutamic dehydrogenase at the end of pregnancy and for the changes in succinic dehydrogenase and cytochrome oxidase found by Moore & Nelson (1952).

Transaminases occur in both supernatant and particulate fractions, predominantly in the supernatant (Nakada & Weinhouse, 1950; Hird & Rowsell, 1950). Glutamic–aspartic transaminase would, therefore, not be expected to follow the same course as the mitochondrial enzymes. That transaminase activity increases as milk secretion increases seems to indicate that transaminase is connected with milk-protein synthesis, but such a connexion might be indirect. All that the increase in transaminase does indicate is an increased turnover of amino groups. Indeed Copenhaver, Shipley & Meyer (1951) have found a striking increase in liver transaminase in diabetic rats, a condition favouring deamination.

The question arises whether all mitochondrial enzymes increase, or only those concerned with the process of milk formation. Two more mitochondrial enzymes were therefore studied, one, cathepsin, probably connected with protein synthesis, and the other, β-glucuronidase, almost certainly not involved in protein metabolism in the rat.

Of the mammary enzymes so far assayed, the increase of cathepsin most clearly parallels the rate

of milk secretion in that it is both rapid and progressive. It is possible to envisage a role for cathepsin in protein synthesis either in terms of the 'template' hypothesis of Haurowitz (1950) or in the transpeptidation hypothesis of Fruton (1951). More recently, Rothschild & Junqueira (1951) have suggested a direct correlation between cathepsin activity and protein synthesis by a reversible proteolysis.

A further possible function of cathepsin has, so far, not been considered. It is possible that cathepsin plays a purely degradative role in maintaining a supply of free amino acids, which are then available for protein synthesis by another system. This explanation would have the virtue of postulating only one function for cathepsin both during the phase of active protein synthesis and during mammary involution, when degradation of the tissue proteins is proceeding. This conclusion would fit in with the results of Page, Glendening & Parkinson (1951) on the changes of cathepsin in the endometrium during the menstrual cycle and with unpublished results of our own on the growing uterus in pregnancy and in uterine involution where an increasing level of cathepsin during uterine growth was further increased during the involutionary phase.

The cathepsin activity clearly does not follow the same pattern of changes as the other mitochondrial enzymes that have been measured, e.g. cytochrome oxidase and succinic dehydrogenase, and it must be concluded that the enzymic make-up of the mitochondria is not constant. This view is borne out by the results obtained with β-glucuronidase.

Although a rough relationship seems to exist between the level of β-glucuronidase during pregnancy and the oestrogen metabolism of the gland, it is difficult to account for the high level of the enzyme during lactation and the peak during involution when oestrogen stimulation is presumed to be diminished. It is worth noting, however, that a similar rise in β-glucuronidase occurs in the involution of placentomatas (Stafford, 1949).

Reviewing the mitochondrial enzymes studied in the mammary gland we thus have three enzymes, succinic dehydrogenase, cytochrome oxidase and glutamic dehydrogenase, which follow one pattern, showing a more or less constant activity during lactation; one enzyme, cathepsin, which increases progressively throughout lactation; and one enzyme, β-glucuronidase, which decreases throughout lactation. It thus appears that mitochondria can have different enzymic constitutions.

It is suggested, therefore, that during the lactation cycle there is a constant turnover of mitochondria and that as these are regenerated at each stage of the cycle they are regenerated complete with those enzymes adapted to the metabolic requirement of the cell at that time.

In view of these considerations it is clearly desirable to extend this study to isolated mitochondria and to attempt to correlate the changes in enzyme levels with the physiological changes of the mammary gland.

SUMMARY

1. Changes in the activity of glutamic dehydrogenase, glutamic–aspartic transaminase, cathepsin and β-glucuronidase have been studied in the mammary gland of the rat at various stages during pregnancy, lactation and mammary involution.

2. The activities of glutamic dehydrogenase and glutamic–aspartic transaminase increase only slightly during pregnancy, but rise sharply after parturition to a high level which is maintained throughout lactation, falling precipitously after weaning.

3. β-Glucuronidase activity increases sharply between the 15th and 20th days of pregnancy but then falls progressively over the period of lactation. There is a dramatic increase in β-glucuronidase after weaning during the period of mammary involution.

4. The catheptic activity of the mammary gland increases progressively from the 10th day of pregnancy up to the 20th day of lactation and increases even further during mammary involution.

5. The results are discussed in relation to possible changes in the mitochondria during the lactation cycle and the possible function of these enzymes in providing precursors for the synthesis of milk protein.

We wish to acknowledge our debt to Dr S. J. Folley, F.R.S., for his interest and advice. Acknowledgement must also be made to Mrs E. I. Crapnell for her able technical assistance. We wish to thank the Royal Society for a grant to one of us (A.L.G.), for the purchase of animals.

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Multiple Components in Horse-Radish Peroxidase

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The horse-radish (*Cochlearia armoracia* L.) is the source of an active peroxidase which has been the subject of many studies. These studies have comprised both accurate work on the kinetics and spectroscopy of the purified enzyme (Keilin & Hartree, 1951; Chance, 1951), and work on the chemical action of relatively crude enzyme extracts. Theorell (1942) first crystallized horse-radish peroxidase, and subsequent workers have used methods of preparation which are substantially modifications of his methods. Although Theorell showed that a substantial portion of the enzyme ('Peroxidase I') was different in electrophoretic and other properties from the material which he finally crystallized ('Peroxidase II'), this finding has been generally neglected. Subsequent workers have tended to assume that their specimens of crystalline peroxidase are a representative sample of the native enzyme of the horse-radish. Keilin & Hartree (1951) go so far as to deny the existence in nature of any component of horse-radish peroxidase other than the major one.

None the less, horse-radish peroxidase is described in textbooks as a typical example of an enzyme with more than one component (see, for example, Sumner & Somers, 1947). In the course of other work it seemed desirable to compare multi-component enzyme systems from *Aspergillus oryzae* (Gillespie, Jermyn & Woods, 1952) with this apparently well-authenticated example of the natural coexistence of enzymes of the same specificity. A brief account of an investigation which showed the existence of four components in horse-radish peroxidase was published by Jermyn (1952*a*). Kondo & Morita (1953) have recently isolated from the sweet potato two peroxidases, *a* and *b*, by differential adsorption on alumina gel. Their peroxidase *a* was similar in properties to horse-radish peroxidase II; the properties of peroxidase *b*, although sharply differentiated from those of peroxidase *a*, were also quite distinct from those reported for horse-radish peroxidase I by Theorell.

Filter-paper chromatography as used by Jermyn (1952*a*) demanded both partial purification of the enzyme and a rigid control of the conditions of development of the chromatogram. A certain amount of trial-and-error adjustment of enzyme concentration was necessary to show up the various components. There was thus always room for the suspicion that some form of artifact had been produced. We have therefore used the method of filter-paper electrophoresis (Cremer & Tiselius, 1950) which could be applied to untreated horse-radish juice. The enzyme system has thus been investigated in a state as little removed as possible from that existing *in vivo*, and the application of any methods of purification has been deliberately avoided.

EXPERIMENTAL

Horse-radish juice

The peeled but unwashed roots were crushed in a Carver laboratory press and the expressed juice was collected and spun in a centrifuge (30 min. at approximately 1000 *g*) until the supernatant liquor was clear. The clarified juice was stored at 5°. No attempt was made to sterilize it, since horse-radish juice is strongly bactericidal (Foter, 1940).

Fig-latex serum

Incisions were made in the outer bark of a Moreton Bay fig tree (*Ficus macrophylla* Roxb.). The latex which escaped was collected and centrifuged in a high-speed head (30 min. at 25000 *g*). The slightly cloudy serum was separated and centrifuged again (15 min.) until clear.

Filter-paper electrophoresis and chromatography

An outline of methods used has been given by Gillespie *et al.* (1952). The technique of electrophoresis is discussed more fully by Woods & Gillespie (1953) and that of chromatography by Jermyn (1953). Electrophoresis was normally carried out at 5–10° on strips of Whatman 3MM paper (40 × 4 cm.) at a potential gradient of 9 v/cm., the initial spot being formed by the application of a volume of 20 μl. to the paper.

The technique for producing 'positive' filter-paper strips by oxidizing with HIO_4 and coupling the resulting carbonyl groups with Girard's reagent *P* has been outlined by Jermyn & Thomas (1953). In order to produce papers with consistent properties, the papers were invariably oxidized by 0·1 M-HIO_4 in the dark at 28°. For the enzymes of horse-radish juice the best patterns were obtained when the paper strips were 6 cm. above the level of the liquid in the electrode vessels.

Spraying reagents

A spraying reagent to detect peroxidase on paper must give a strong colour over a wide range of enzyme concentrations under conditions where an excess of H_2O_2 is present, since very dilute H_2O_2 solutions are too readily decomposed on the paper to be effective. Benzidine and guaiacol were found to be the most satisfactory for this purpose. Saturated, aqueous solutions of benzidine acetate or guaiacol were mixed with 0·01 vol. of 30% (w/v) H_2O_2 soln. Under favourable conditions the guaiacol red was stable for some hours; the benzidine blue faded within a few minutes at high enzyme concentrations. The benzidine action is inhibited on the paper at pH values above 7·5. Other, somewhat less satisfactory, reagents were *p*-aminodimethylaniline (reddish violet), *o*-anisidine (blue-violet) and hydroquinone (brown).

In general there was a critical threshold concentration of enzyme for the appearance of a component on spraying; components visible after electrophoresis as discrete spots when the horse-radish juice was applied directly were often invisible when it was applied after dilution.

Determination of peroxidase

After electrophoresis the paper strip was cut transversely into pieces of 0·5 cm. width which were placed serially in 6 × $\frac{5}{8}$ in. test tubes and covered with 2 ml. of 0·1 M sodium acetate buffer (pH 5·0) for an hour at room temperature. To each tube were added 3 ml. of a mixture of equal volumes of saturated guaiacol solution, 0·1 M-H_2O_2 solution and water. After incubation for 10 min. at 37°, 2 ml. of N-NaOH were added and the optical density at 400 mμ. was measured in a Coleman Universal Spectrophotometer. The greenish-yellow colour was stable for at least 24 hr. There is a linear relationship between optical density and enzyme concentration up to an optical density of about 0·8. Optical density at 400 mμ. may therefore be taken as a measure of the enzyme extracted from the paper pieces; in certain cases, however, the enzyme was found to be tenaciously adsorbed by the paper. Incubation with guaiacol and H_2O_2 then led to strong development of colour on the paper, little or none developing in the solution. Addition of alkali extracted the red colour from the paper to give a very faintly yellow solution. Paper pieces carrying adsorbed enzyme thus give a falsely low or zero reading for their enzyme content. Direct spraying of the entire strip is therefore the only valid measure of enzyme distribution. Determination of this distribution by extraction methods is no more than a rough quantitative guide to the relations between those components that can be readily extracted from the paper.

Precipitation with picric acid

In experiments where precipitation with picric acid was studied, horse-radish juice, water and saturated picric acid solution were mixed in a centrifuge tube in the required proportions and centrifuged till the supernatant was clear. The clarified supernatant was then investigated electrophoretically.

RESULTS

The existence of multiple components

We have found that the most satisfactory ionic environment for the separation of horse-radish peroxidase into distinct components is 0·1 M sodium acetate buffer at pH 5·0. The enzyme is quite stable on the paper in the pH range 5–8, but outside these limits considerable inactivation takes place. This inactivation is most marked on the acid side and at pH 3, where rapid splitting of peroxidase is known to take place (Maehly, 1951); the survival time of the enzyme is very short. As the pH of the buffer is raised above 5·0, or its strength is decreased below 0·1 M, electro-osmotic flow towards the cathode increases and the resulting displacement of the pattern produces a much smaller separation of the anodic components from the adsorbed cathodic material; nevertheless, for certain experiments where rapid migration was important, we have used 0·01 M sodium acetate buffer.

Using the standard conditions and untreated paper strips, electrophoresis of freshly expressed horse-radish juice (for a period of about 24 hr.) gives a pattern which is shown diagrammatically in Fig. 1. The anodic components (A_1 and A_2) appear as discrete spots, but the cathodic components (C_1 and C_2) appear as well-defined streaks of complicated structure over which the neutral (N) component is superimposed by electro-osmotic flow. The cathodic appearances are similar to the phenomena which Weber (1953) called 'comets', for which he has given a theoretical explanation.

It has been possible to resolve the confused pattern on the cathodic side by the use of 'positive' papers. We have previously shown (Jermyn & Thomas, 1953) that, as the time of periodic acid oxidation is lengthened and the number of substituted pyridinium groups correspondingly increased, the normal cathodic electro-osmotic flow at pH 5 slows down, becomes zero ('neutral' paper), and reverses direction, becoming increasingly anodic ('positive' papers). Polyelectrolytes with a net positive charge, which are absorbed by the untreated papers (weakly 'negative' under the conditions of these experiments at pH 5), become free to move in the electric field on 'positive' papers; negatively charged polyelectrolytes however will show exactly opposite behaviour.

The results of experiments using 'positive' papers are shown in Fig. 2. All the main features of these photographs can be explained in terms of the principles just stated. Thus it is to be expected that the cathodic components will show the observed maximum migration towards the cathode in view of the opposite effects of decreased adsorption and increasingly anodic liquid flow.

We have also carried out some experiments using bromine-oxidized 'negative' (Jermyn & Thomas, 1953) papers at pH 5·0. *N* here moves rapidly towards the cathode quite clear of C_1 and C_2, which are very strongly adsorbed. By using sufficiently negative papers where the cathodic liquid flow is several cm./hr. under our conditions, A_1 and A_2 can be brought to lie cathodically to C_1 and C_2, although still on the anodic side of the position occupied by a neutral indicator.

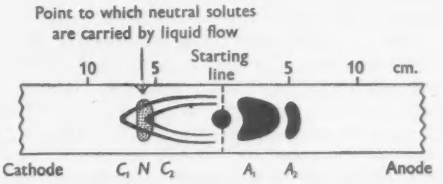

Fig. 1. Diagrammatic representation of the distribution of peroxidase components in freshly expressed horse-radish juice after 24 hr. electrophoresis on untreated paper at pH 5·0 in 0·1 M sodium acetate buffer.

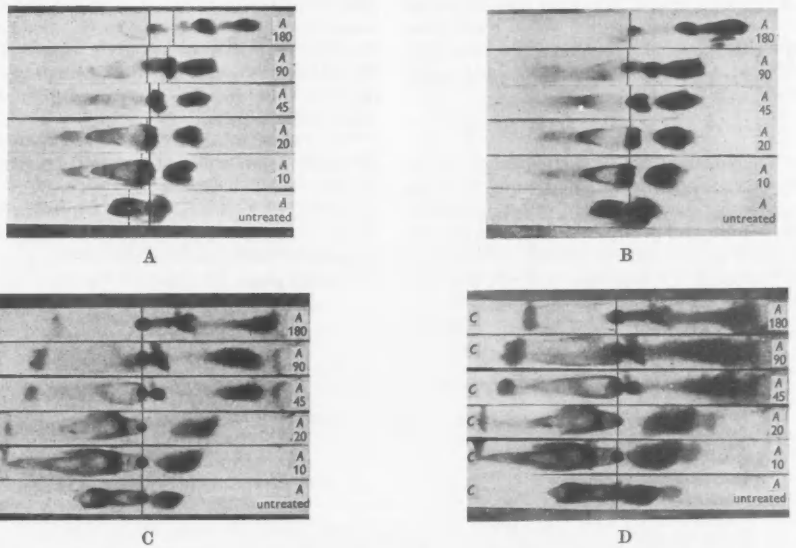

Fig. 2. Filter-paper electrophoresis of freshly expressed horse-radish juice in 0·1 M sodium acetate buffer at pH 5·0. *A* = anodic end of strip; the numbers (10, 20, 45, 90, 180) indicate the time in minutes for which the paper strips were oxidized in 0·1 M-HIO_4 at 28° before treatment with the Girard reagent *P*. A, six hr. electrophoresis; sprayed with the guaiacol reagent. Broken lines indicate the position to which neutral substances are carried by liquid flow. B, the same after further spraying with benzidine reagent. C, a different sample after 15 hr. electrophoresis; sprayed with the guaiacol reagent. D, the same after further spraying with the benzidine reagent. Photographic reproduction does not do justice to the immediate visual appearances, since considerable diffusion and blurring is inherent in the double spraying of wet papers, besides the fogging that occurs as traces of enzyme show up with the lapse of time.

No colour was developed in the positions of any of the components on spraying with a reagent containing benzidine or guaiacol alone in the absence of H_2O_2. The peroxidase activity was totally and irreversibly destroyed by heating. Hence none of the components was an oxidase or an inorganic catalyst being revealed as an apparent peroxidase by our technique of detection.

Anodic components

The two anodic components have very distinct properties. These are set out in Table 1. The concentration of A_2 is rarely high enough for it to react with guaiacol on the paper; guaiacol-sprayed papers therefore reveal A_1 alone. A subsequent spraying with the benzidine reagent brings up A_2 and we have regularly used this method to differentiate between the two components (cf. Fig. 2D). A_1 is negatively charged at pH 5·0, as shown by its migration towards the anode; rapidly increasing inactivation in experiments at pH values much below this has prevented us from determining the isoelectric point. In keeping with this negative charge, A_1 shows an increasing tendency to trail as more positive papers are used. Under certain conditions A_1 shows a tendency to split into two components, the slower moving of which reacts less intensely with guaiacol. However, we have not been able to establish definite conditions for bringing about this separation.

Cathodic components

Three components may be distinguished in the material with an apparent cathodic migration on untreated paper. One is nearly at its isoelectric point, at pH 5·0 and occupies almost exactly the same position as the neutral indicator substances (trinitrobenzene and *o*-nitroaniline) used to determine the direction and amount of liquid flow. At pH 7·5 it is negatively charged and migrates anodically behind A_1. On highly positive paper both this component N and the anodic components migrate much faster than would be expected from the apparent extent of anodic liquid flow (Fig. 2, 180 min.). Thus the simple model which we have used to interpret our observations does not account for the mobility of proteins on highly charged papers. N is almost always the major single component present; only during the autumn of 1952 was there more of A_1 than of N.

Of the two cathodic components, Fig. 2 shows that the individuality of C_1 is most readily demonstrated. It is very readily inactivated on storing the expressed juice, or on very positive papers as shown by the disappearance of this component on the 90 and 180 min. papers in Fig. 2, A and B, where the sample of juice used was low in C_1. C_1 shows an almost specific reactivity with *o*-anisidine, none of the other components giving more than a faint reaction with this reagent under our conditions. The absorption of C_1 is very slight and it moves relatively freely on 'neutral' papers.

It is doubtful what significance is to be attached to C_2, which includes the rest of the cathodic material. The plate shows that even on quite positive papers there are multiple fronts and obvious adsorption in the region which C_2 occupies. None the less, on the most positive papers (180 min.), this cathodic trailing has disappeared and a fairly distinct spot can be seen immediately on the cathodic side of N.

In contrast to A_1, A_2 and N which are quite unaffected in mobility by the ageing of a given sample of juice, or by variations between different samples, C_1 and C_2 give patterns which are strongly affected by these factors. Whole series of overlapping fronts appear and disappear and the apparent mobility on untreated paper alters markedly. These phenomena always disappear as increasingly positive papers are used.

Table 1. *Comparison of the properties of the two anodic components of horse-radish peroxidase*

	Slow anodic component (A_1)	Fast anodic component (A_2)
Reaction with various spray reagents:		
Benzidine	+ + +	+ +
Guaiacol	+ +	±
o-Anisidine	−	−
Hydroquinone	+	−
p-Aminodimethylaniline	+ + +	+ +
Adsorption on untreated paper	Slight	Nil
Approximate mobility at pH 5·0 in 0·1 M sodium acetate buffer (cm.²/v/sec.)	$1{\cdot}0 \times 10^{-5}$	$1{\cdot}5 \times 10^{-5}$
Seasonal variation	Absent in summer	Invariably present
Concentration	Major component in winter	Always a minor component
Homogeneity	Sometimes shows incipient splitting into two spots	Always a single spot

Immobile material

A certain amount of immobile material is always apparent occupying an area corresponding exactly to that of the original spot of juice. The phenomenon seems to be due to the precipitation of non-enzyme material on the paper followed by secondary adsorption of the enzyme, since the adsorbed material gives all the colour reactions of the mobile components. It is interesting to note that esterase, which is detected by a diacetylfluorescin spray reagent and is the only other enzyme discovered in horse-radish juice, forms a single immobile spot under all conditions of electrophoresis.

Seasonal variations

Since the peroxidase content of horse-radish roots varied according to soil, weather and season, arrangements were made to obtain regular small samples of roots from a permanent bed maintained in a market garden. The following variations were noted: (*a*) Peak peroxidase activity occurs in autumn–winter (May 1952; June 1953). After this the activity declines to a minimum in early summer (December). (*b*) The cathodic components (N, C_1 and C_2) are always present, but show a minimum in summer. (*c*) A_2 is less affected by seasonal variation than any other component. (*d*) A_1 is completely absent at the peroxidase minimum (summer) and is present in maximum amount at peroxidase maximum (winter). (*e*) The concentration of peroxidase present at the maximum varies from season to season. Thus in 1952 the juice had to be diluted with water (1:2, v/v) to obtain reasonable patterns; in 1953 it could be run undiluted (1952 was a very wet autumn, 1953 a very dry one).

Reaction with picric acid

One of the points of difference between peroxidases I and II noted by Theorell was that peroxidase I was precipitated by picric acid and peroxidase II was not. We have observed that very little peroxidase activity is precipitated from absolutely fresh horse-radish juice by picric acid, unless the juice was diluted. This is true even when the picric acid concentration is high, e.g. for mixtures of one part of juice with two parts of saturated picric acid solution. Neither of the two major components (A_1 and N) is at all readily precipitated by picric acid and this property is almost entirely confined to the cathodic components C_1 and C_2. We have found that these minor components are usually precipitated by an equal volume of saturated picric acid, although dilution with water is necessary with samples taken at the period of maximum activity.

Ageing of horse-radish juice

It has been found that intact horse-radish roots can be stored in the refrigerator at 5° for some weeks without any change in the electrophoretic pattern of the juice when expressed. The changes observed in successive samples of fresh material appear therefore to be associated with the continuance of normal growth processes. When undiluted horse-radish juice was stored at 5° there was little or no apparent change in any of the components for about 3 weeks. After this time A_1 began to lose activity and become increasingly precipitable by picric acid. The changes on storage are illustrated by the histograms of Fig. 3.

The juice used in these experiments was expressed during the 1952 maximum of peroxidase activity, when it had to be diluted (1 in 3) to obtain patterns with discrete spots. The standard mixture for test of

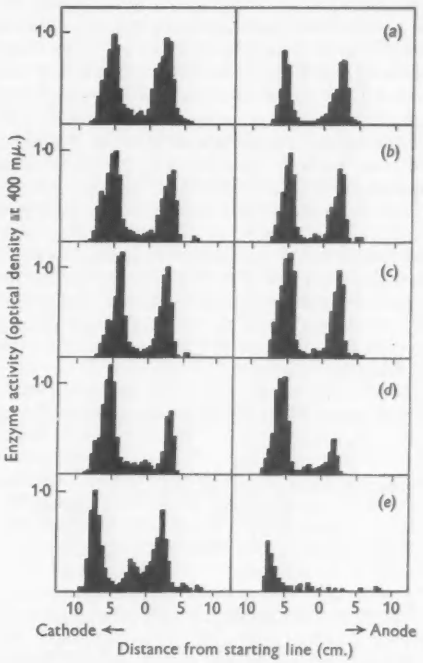

Fig. 3. Histograms of the distribution of horse-radish juice peroxidase on filter-paper electrophoresis (pH 5·0, 24 hr. at 6 v/cm., 0·1 M sodium acetate buffer) after various times of storage. Activities measured by the guaiacol: alkali method. Left-hand side, sample diluted to 1 in 3 with water; right-hand side dilution; sample : sat. picric acid solution : water (1:1:1, v/v). (*a*) Sample 2, fresh juice; (*b*) sample 2, 4 days storage; (*c*) sample 2, 11 days storage; (*d*) sample 2, 25 days storage; (*e*) sample 1, 40 days' storage.

picric acid precipitability was, therefore, equal volumes of juice, saturated picric acid solution and water. The diluted juice and the supernatant from the picric acid precipitation could thus be compared directly.

C_1 and C_2 do not show up as significant peaks on histograms of fresh material, and observation shows them to be adsorbed strongly on the paper. The appearance of more than one peak on the cathodic side of the histograms of aged material is due to less firm adsorption on the paper, and the enzyme reaction can now be seen to take place not only on the surface of the paper but in the reagent solution as well.

Re-running of isolated components

Objection is commonly made to the reality of multiple components such as those we have observed, on the grounds that they are artifacts of the process of separation, and such artifacts are in fact well-known from paper chromatography (Smith, 1952; Boman, 1952; Moore & Stein, 1952). Such objection can usually be met by isolating and purifying individual components and subjecting them to the original experimental conditions; if these isolated components show no signs of splitting into the multiple components of the original experiment they are adjudged to have real existence. In order to meet objections that have been strongly urged against the idea of multiple peroxidase components on other grounds (Keilin & Hartree, 1951) we were anxious to avoid any isolation or purification of individual components that involved the usual chemical and physical manipulations. We have therefore resorted to the following expedient.

A peroxidase component which has moved into an area of paper which is entirely separate from the areas occupied by other components is effectively 'isolated'. It is impracticable to elute this component and use the resulting solution of the 'pure' component to provide the initial spot for a new electrophoretic run, since quite a high minimum concentration of enzyme is necessary to leave sufficient activity to be detected by our method. We have therefore removed the area of paper containing the desired component (located by comparison with a second control strip) and placed it at the exact centre of the glass plate. Fresh paper strips saturated with buffer were placed in contact with it and a new electrophoresis was begun. The process is illustrated for A_2 in Fig. 4. We have been able to demonstrate that A_1, A_2 and N each continue their normal migration into the fresh strips with approximately the same mobility as before. There is no tendency to give rise to any of the other components. A_2, for instance, continued to migrate rapidly into the anodic strip, leaving the original area it occupied devoid of enzyme activity, and giving rise at no stage to guaiacol-sensitive material.

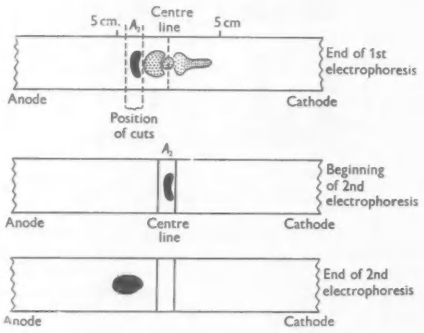

Fig. 4. Diagrammatic illustration of experiment demonstrating that A_2 is a pure component. Electrophoresis carried out in 0·01 M sodium acetate at pH 5·0. Duration of first run, 2 hr.; of second run, 4 hr.

Since perhaps only 0·1% of the material in the original horse-radish juice was peroxidase it was possible that the isolated components might run differently in the absence of most of the material with which they had been originally associated. The areas containing isolated components were therefore dipped into horse-radish juice that had been boiled till peroxidase activity was just destroyed and a second electrophoresis was carried out as before. There was no perceptible difference in the results. A certain amount of material was thrown out of the horse-radish juice on boiling and had to be removed by centrifuging.

Fig peroxidase

Fresh fig-latex serum (diluted 1 in 3) was run at pH 5·0 under the same conditions as horse-radish juice. The electrophoretic pattern is shown by the histogram of Fig. 5. This was confirmed by spraying, which showed an intense anodic spot with a decreasing trail back to a faint spot of absorbed material at the starting-point.

The fig peroxidase was completely removed by picric acid at a ratio of two parts of serum to one part of saturated picric acid solution; under parallel conditions very little of the fresh horse-radish peroxidase was precipitated. It is thus apparent that fig peroxidase is a protein of different properties from those of the horse-radish enzymes. Its amount is subject to very little seasonal variation; the only other component that we have been able to detect is a very small quantity of strongly adsorbed cathodic material, in early spring. It should be possible to obtain a single peroxidase from fig latex which is much more representative of the total

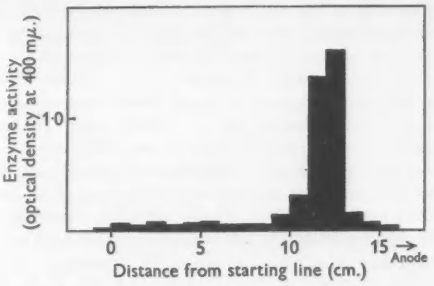

Fig. 5. Histogram showing the distribution of the peroxidase of fig-latex serum (diluted 1 in 3) after filter-paper electrophoresis (pH 5·0, 24 hr. at 6 v/cm. 0·1 M sodium acetate).

peroxidase of the source than that usually obtained from the horse-radish.

When fig-latex serum and horse-radish juice were mixed (2:1) and run at pH 5·0, the anodic spot of the fig peroxidase segregated quite independently of the pattern due to the horse-radish enzyme. Its position was exactly the same as that from a serum diluted 2:1 with water and run in parallel.

Other plant peroxidases

A single experiment on the peroxidases of the expressed juice of the sweet potato (*Ipomaea batatas* Poir.) and the swede turnip (*Brassica napobrassica* Miller) carried in early spring (September) showed in each case the presence of major anodic components of mobility similar to that of A_2, which were reactive with both guaiacol and benzidine. The sweet potato further showed a strongly adsorbed, slower, anodic component and a benzidine-sensitive component which was approximately neutral. The turnip, in addition to the major component, showed only a single, slowly moving, strongly adsorbed cathodic component, sensitive to both benzidine and guaiacol.

DISCUSSION

Nature of the components

The evidence presented in this paper and that of Jermyn (1952*a*) seems enough to justify the conclusion that multiple components are present in the native peroxidase of the horse-radish. However, since the existence of multiple components in naturally occurring enzymes is far from being generally accepted, it is essential to consider other possible explanations of the observed phenomena. The following hypotheses may be examined:

(*a*) The observed components are artifacts of the technique of electrophoresis. Our experiments in re-running isolated components seem to rule out the occurrence of 'double fronting' of the type found by Boman (1952) and Smith (1952) in paper chromatography. In any case the small amount of ions applied in the spot seems insufficient to produce lasting 'fronts' of different ionic concentrations during a long enough period to affect the electrophoresis, and to the best of our knowledge such effects have never been observed in filter-paper electrophoresis.

(*b*) The observed components are actually a series of complexes between a single peroxidase and a number of complexing agents present in horse-radish juice. Our observations show that these complexes must be sufficiently tightly bound to migrate as discrete spots, to undergo a second electrophoresis without regenerating any other 'peroxidases' and to exhibit distinctly different specificity for a range of substrates. Even flooding the area of paper occupied by a single component with boiled horse-radish juice does not cause these components to break up into multiple components. Hence the postulated complexes must be formed irreversibly and non-competitively and it is difficult to see how they are to be operationally distinguished from separate enzymes. It is possible that the complexing substances occur in the material coagulated by heat. There is evidence against this from the independent development of the characteristic peroxidase patterns of horse-radish juice and fig serum when a mixture of the two is subjected to electrophoresis. Since all the heat-coagulable substances in both materials are still present, it appears that there can be no free complexing agent in either which is capable of reacting with the peroxidase of the other. It is common experience that the electrophoretic mobility of proteins on paper is affected by the presence of other proteins, and this point has been shown specifically for interactions with polyglutamyl peptides by Strange & Harkness (1953). None the less, the effect of such loose interactions is to blur true separations rather than to create new ones (E. F. Woods, private communication). If our components are complexes, then their behaviour is exactly parallel to that of mixtures of chemically distinct, simple substances, rather than to that of the vague and shifting complexes studied by Hall & Wewalka (1952) and Franklin, Quastel & Van Straten (1952) using paper chromatography. Fišer-Herman & Davorin (1953) have shown the sharp segregation of individual haematin-protein complexes on filter-paper electrophoresis even in such a complicated mixture as animal serum.

(*c*) If the observed components are actually tightly bound complexes, it is possible that they are in fact one and the same haemoprotein with the sixth co-ordination position of iron filled by different ligands. This is the explanation advanced by Keilin

& Hartree (1951) to account for the difference between 'peroxidase' and 'paraperoxidase' which they identify with Theorell's peroxidase I. We observe changes in the colour of horse-radish juice on standing, the original brownish yellow turning to red; such changes are characteristic of alterations in the co-ordination sphere of the iron atom and are found by Keilin & Hartree in the conversion of peroxidase solutions into paraperoxidase solutions on standing. We do not find that colour change in the horse-radish juice is accompanied by any changes in the electrophoretic mobility and it seems most unlikely that such alterations at a single active point in the enzyme could have such a striking effect on the isoelectric point and electrophoretic mobility of the molecule. Furthermore, we have attempted to carry out our experiments in such a way that changes in the haem portion of the molecule are minimized.

(*d*) The same haemoprotein may be modified by conjugation with other proteins or the addition of non-protein acidic and basic materials to the molecule. The presence of an acidic polysaccharide as part of the molecule of peroxidase II was invoked by Theorell & Åkeson (1942) to explain the differences between peroxidases I and II. The activity of such conjugated enzyme proteins against a given substrate should be nearly equal; Theorell (1940), in fact, found nearly equal activities for peroxidases I and II. There is no evidence available at present by which it could be decided whether the range of specificities we have encountered in our components is compatible with this hypothesis.

(*e*) The observed components may be separate enzymes, unrelated except that they are all haemoproteins. Kondo & Morita (1953) have shown the existence of electrophoretically distinct peroxidases in the sweet potato, one of them with properties different from any peroxidase described for the horse-radish. Different enzymes of *A. oryzae* occurring in the same mixture (Gillespie *et al.* 1952) show patterns of single or multiple components which are completely independent of each other on electrophoresis under identical conditions. The fig and horse-radish peroxidases and so far as our limited experiments go, turnip and sweet-potato peroxidases also, give patterns at least as different as those of distinct enzymes in *A. oryzae*. Such differences in the electrophoretic mobility of the same enzyme from botanically distinct species would normally be unhesitatingly ascribed to the existence in these species of enzyme proteins of specific properties carrying a universal prosthetic group. There is no reason to suppose that the horse-radish itself could not produce a number of different peroxidase proteins, possibly from separate tissues of the plant. Van Fleet (1947) has demonstrated that peroxidase is differently distributed in the tissues at succeeding stages in the development of the horse-radish and claims that the peroxidase of different tissues is not of identical properties.

The independent seasonal variation of our components is a point in favour of their being separate enzymes. Gillespie *et al.* (1952) and Jermyn (1952*b*) have demonstrated the separate nature of the β-glucosidase components of *A. oryzae* by showing that one or more of them may be absent from independently prepared samples. Gillespie & Woods (1953) have made a similar deduction for the proteases of *A. oryzae*.

The identity of the components

One piece of evidence allows the probable identification of one of our components. A sample of highly purified crystalline peroxidase (peroxidase II) was obtained through the courtesy of Prof. H. Theorell and a suitably diluted solution was subjected to electrophoresis under our standard conditions. The electrophoretic pattern consisted of a single intense spot identical in position and behaviour with component N_1 plus a little adsorbed material appearing as a faint diffuse trail and a trace of anodic material. Peroxidase II, negatively charged at pH 7·5 (Theorell, 1940), is thus apparently nearly isoelectric at pH 5·0; peroxidase I, already positively charged at pH 7·5, must have a still greater tendency to move to the cathode at pH 5·0. If we identify N with peroxidase II then, of the components present in our experiments, peroxidase I could only be represented by either or both of C_1 and C_2. This possibility is confirmed by the relative ease of precipitation of these components with picric acid, but it would be most unwise, in view of the doubts that have been cast on the real existence of peroxidase I, to make a positive identification on the present evidence. Components A_1 and A_2 do not appear to correspond with anything that has hitherto been reported. It is noteworthy that we have observed major anodic components, carrying a strong negative charge at pH 5, in the fig, turnip and sweet potato.

Peroxidases I and II were originally thought to be of equal importance in the horse-radish and the turnip (Theorell, 1940). The reported seasonal variation in peroxidase I and the difficulty of purifying it have led to its being little studied in comparison with peroxidase II which has usurped the name 'peroxidase'. An agreed terminology appears to be necessary for discussion of the subject—the supposed identity of the 'paraperoxidase' of Theorell (1947) and that of Keilin & Hartree (1951) appears to consist of little more than an identity of labels. The following is suggested. The term 'peroxidase' is to be equally applicable to all native plant enzymes with peroxidatic activity.

The absorption spectrum of the haemoprotein or the nature of the ligands co-ordinated with it would then be irrelevant. All the components revealed by this study would then be 'peroxidases' if they are indeed separate, naturally occurring species. The term 'paraperoxidase' with its implication of the modification of an original enzyme could be applied in the sense of Keilin & Hartree (1951) to the insoluble or less-soluble derived materials formed from peroxidases on storage. Component A_1 may thus have formed a 'paraperoxidase' in our experiments on the ageing of horse-radish juice.

SUMMARY

1. The peroxidases of horse-radish juice and the latex serum of *Ficus macrophylla* have been examined by filter-paper electrophoresis.

2. Horse-radish contains possibly five components with peroxidase activity; *Ficus macrophylla*, one.

3. There is great seasonal variation in the relative amounts of the peroxidase components present in horse-radish juice; one of the two major components is absent in the late spring.

4. A major component is always present in horse-radish juice which corresponds to the peroxidase II of Theorell; other material probably corresponding to his peroxidase I is also present.

5. Two components have been discovered which are negatively charged at pH 5·0 and cannot be equated with either peroxidase I or II.

6. One of these components has a positive peroxidatic activity against benzidine and little or none against guaiacol; all the components show some differences in reactivity towards various substrates.

We wish to thank Prof. H. Theorell for a gift of crystalline peroxidase, and Mr H. V. Barnett of Moorabbin, Melbourne, for making available a regular supply of horse-radish.

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A Colorimetric Micro-method for the Determination of Glucose

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When a dilute solution of glucose is heated with concentrated sulphuric acid, a bluish pink colour develops, the intensity of which is proportional to the concentration of glucose (Mendel & Bauch, 1926). This reaction has served as the basis for simple methods of determining glucose in blood (Mendel & Bauch, 1926; Mendel & Hoogland, 1950) and glycogen in tissues (Kemp & Kits van Heijningen, 1954). Both methods depend on the formation of 5-hydroxymethylfurfural (HMF) from glucose in hot acid, a reaction which has been studied by Holzman, MacAllister & Niemann (1947), Singh, Dean & Cantor (1948), Wolfrom, Schuetz & Cavalieri (1948) and Ikawa & Niemann (1949). The coloured compound seems to be produced in a subsequent reaction between HMF and one of its pre-

cursors. This reaction is highly dependent on the concentration of sulphuric acid and appears to be specific for glucose, fructose and related carbohydrates.

EXPERIMENTAL

Reagents

Sulphuric acid. The only reagent necessary for the determination of glucose in pure solution is concentrated H_2SO_4. The samples of 96% (w/w) H_2SO_4 (sp.gr. 1·84) obtained from Canadian Industries Ltd. (Canada), Grasselli (U.S.A.), Brocades-Stheeman (Holland), Merck and Co. (Germany) and May and Baker (England) proved to be suitable. Samples supplied by a number of other firms often gave a yellowish pink instead of the bluish pink colour. The concentration of H_2SO_4 determined by titration should be approximately 36 N. If the concentration in commercial samples is too high, the H_2SO_4 has to be diluted and should then be allowed to stand 2–3 days in a stoppered bottle before use.

Deproteinizing solution. For determining the glucose concentration in blood samples, the blood is deproteinized with a 5% (w/v) solution of trichloroacetic acid (TCA) (AnalaR Reagent) containing 0·1% (w/v) Ag_2SO_4 (A.R.) (Mendel & Hoogland, 1950).

Procedure

One ml. of an aqueous glucose solution, containing not more than 15 mg. glucose/100 ml., is added to 3 ml. of 96% H_2SO_4 in a wide test tube, the contents of which should be mixed immediately by vigorous shaking. After mixing, the concentration of H_2SO_4 in the solution is 82% (w/w); the temperature of the solution rises temporarily to about 85°. The mixture is heated for exactly 6·5 min. in a boiling-water bath; the water should be boiling vigorously to prevent a decrease in temperature when the test tubes are inserted. The test tubes are subsequently cooled under tap water and the intensity of the pink colour is measured at 520 mμ. in a Beckman Spectrophotometer, model DU. The method can easily be adapted for the Coleman Junior or any other colorimeter. Should it be inconvenient to measure the optical density immediately, the tubes can be stoppered and allowed to stand at room temp. for several hours, since the pink colour is stable in 82% H_2SO_4.

Various modifications of this procedure have been tested but they did not appear to offer any advantage. The same colour intensity is obtained when (*a*) the H_2SO_4 is added to the glucose solution, (*b*) the glucose solution and H_2SO_4 are cooled to 0° while being mixed, and (*c*) the mixture is not heated at 100° immediately but kept for 30 min. before being heated.

For determining the blood-sugar level, 0·1 ml. blood is pipetted into 1·9 ml. of the TCA: Ag_2SO_4 solution. The pipette should be rinsed well with the deproteinizing solution and the suspension mixed briefly. The suspension is centrifuged at 3000 rev./min. for 5 min. to obtain a clear supernatant fluid. The precipitate can also be removed by filtration through a small filter paper (cf. Mendel & Hoogland, 1950), but special care must be taken to ensure that the paper is free of chlorides. One ml. of the clear supernatant fluid or filtrate is added to 3 ml. of conc. H_2SO_4 and the colour reaction is carried out as described above.

The concentration of glucose in the TCA extract can be read from a standard curve. Up to a concentration of 15 mg./100 ml., the relationship between the glucose concentration and colour intensity is linear, an extinction of about 0·160 at 520 mμ. being obtained with an aqueous solution containing 10 mg. glucose/100 ml. The same standard curve can be used as long as the concentration and brand of H_2SO_4 remain unchanged.

RESULTS

Absorption spectrum

Figs. 1 and 2 show the absorption spectrum of the coloured solution which is obtained after heating a dilute solution of glucose for 6·5 min. at 100° in 82% sulphuric acid. Absorption maxima are found at 520 mμ. in the visible range (Fig. 1) and at 318 and 255 mμ. in the ultraviolet (Fig. 2). The absorption peak at 520 mμ. is due to the bluish pink compound which is formed in the reaction. The maxima at 318 and 255 mμ. are due to the formation of HMF from glucose in heated sulphuric acid, their positions and relative optical densities being almost identical with those of HMF dissolved in sulphuric acid of the same concentration (Fig. 2).

Upon dilution of the coloured reaction mixture with water, the absorption maxima in the ultraviolet shift their positions in precisely the same way as do the absorption maxima of HMF. The bluish pink colour diminishes when the sulphuric acid is diluted, and disappears completely when the concentration of sulphuric acid is decreased to approximately 55%. Since the absorption spectrum of HMF is still present under these conditions, it seems probable that the coloured compound dissociates into simpler molecules in less concentrated sulphuric acid.

The bluish pink compound is not formed when HMF is heated with concentrated sulphuric acid, though a slight yellowish colour with a maximal absorption at 430 mμ. is produced. However, the intensity of the pink colour is not diminished when up to 25% of the glucose is replaced by HMF (Fig. 3). It appears, therefore, that the formation of the coloured compound in heated sulphuric acid is dependent on the presence of HMF and a compound intermediate between glucose and HMF.

When the course of the reaction is followed by means of the absorption spectra, the concentration of HMF is found to reach a maximum after approximately 2 min. at 100°, as judged by the optical density at 318 mμ. (Fig. 4). It can be calculated that about 65% of the glucose originally present must have been converted into HMF at this point. With continued heating the HMF concentration decreases. The bluish pink colour appears more slowly than the HMF and reaches its maximum after about 10 min. at 100° (Fig. 4).

Further heating fails to cause an appreciable change in the intensity of the bluish pink colour; however, a brownish colour with an absorption maximum at 430 mμ. appears, the intensity of which increases the longer the glucose is heated with concentrated sulphuric acid (Fig. 4). The heating time of 6·5 min. at 100° was selected to permit a maximal development of the bluish pink colour with minimal interference by the brownish decomposition products.

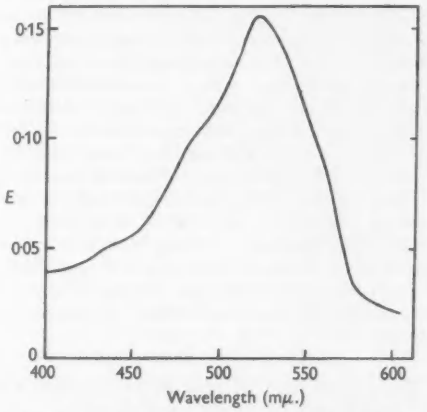

Fig. 1. Visible spectrum obtained after heating 1 ml. of an aqueous glucose solution (0·01 %) with 3 ml. of 96 % (w/w) H_2SO_4 at 100° for 6·5 min. The extinctions were measured in a 1 cm. cuvette with a Beckman Spectrophotometer.

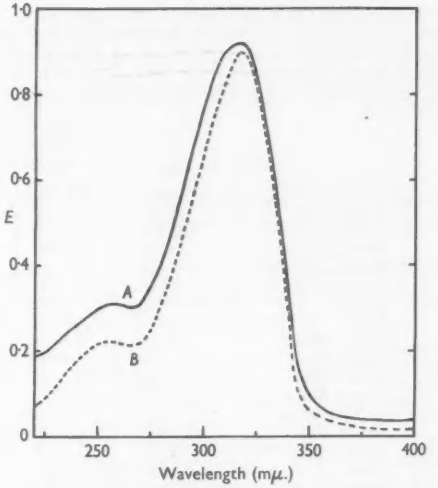

Fig. 2. Ultraviolet spectra obtained (*A*) after heating 1 ml. of an aqueous glucose solution (0·01 %) with 3 ml. of 96 % (w/w) H_2SO_4 at 100° for 6·5 min., and (*B*) after mixing 1 ml. of an aqueous solution containing 29·6 μg. of 5-hydroxymethylfurfural with 3 ml. of 96 % H_2SO_4 at 0°.

Sulphuric acid concentration

Fig. 5 shows the results obtained when a dilute glucose solution is heated for 6·5 min. at 100° in sulphuric acid of different concentrations. Pro-

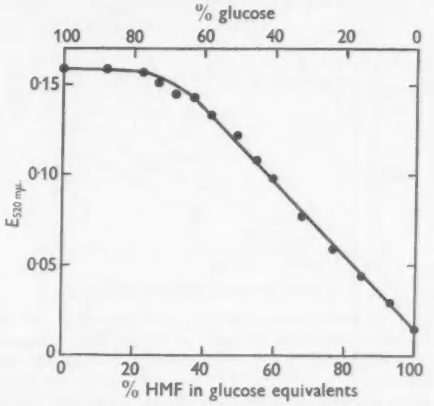

Fig. 3. The extinction of solutions containing glucose plus 5-hydroxymethylfurfural (HMF) in various proportions was measured at 520 mμ. after the solutions had been heated with three times their volume of 96 % (w/w) H_2SO_4 at 100° for 6·5 min. The total concentration of glucose plus HMF in the aqueous solutions was 5·56 × 10^{-4} M, equivalent to 0·01 % glucose.

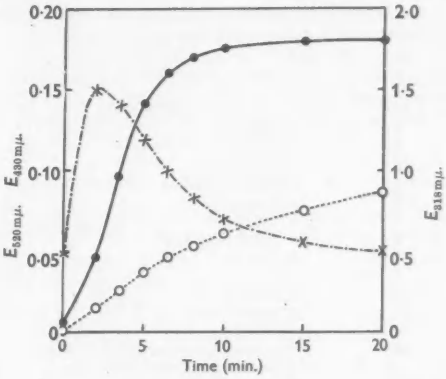

Fig. 4. The extinctions obtained after heating 0·01 % glucose solutions with three volumes of 96 % H_2SO_4 at 100° for various intervals of time were measured at 520 mμ. (●—●), 430 mμ. (○----○) and 318 mμ. (× ·—·— ×). The figures obtained for the extinction at 318 mμ. are plotted on one-tenth of the scale for the values at 520 and 430 mμ.

gressively larger amounts of HMF and brownish decomposition products are formed from the glucose as the concentration of the sulphuric acid is increased. The intensity of the pink colour, on the other hand, reaches a maximum when the solution contains 82–83 % sulphuric acid. When much more concentrated or more dilute solutions of sulphuric acid are used, the pink colour fails to appear.

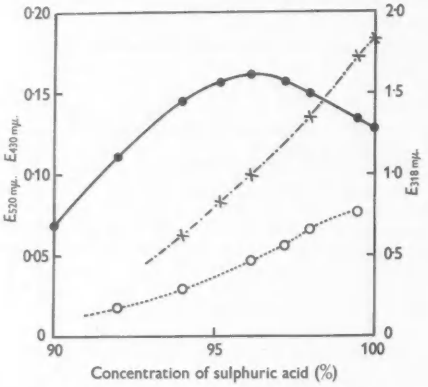

Fig. 5. Glucose solutions (0·01 %) were heated at 100° for 6·5 min. with 3 vol. of H_2SO_4 of various concentrations. The extinctions were measured at 520 mμ. (●—●), 430 mμ. (○----○) and 318 mμ. (×–·–·–×); the figures obtained for the extinction at 318 mμ. are plotted on one-tenth of the scale used for the values at 520 and 430 mμ.

A final concentration of sulphuric acid within the optimum range is conveniently obtained by mixing 1 ml. aqueous glucose solution with 3 ml. of commercial 96 % sulphuric acid. Under these conditions the ratio of the optical densities at 520 and 430 mμ. after heating for 6·5 min. should be about 3 and the resulting solution should have a bright pink colour. However, it has been found that some samples of sulphuric acid may give a considerably lower optical density at 520 mμ. and a higher density at 430 mμ., even though the concentration has been adjusted to 96 %. In this case the ratio of the optical densities at 520 and 430 mμ., after heating with the glucose solution, may be as low as 1·6 and the pink solution will have a distinct brownish tinge. Although this latter type of sulphuric acid is much less suitable for the colorimetric determination of glucose, both types will give a linear relationship between glucose concentration and optical density at 520 mμ.

Specificity of the colour reaction

The colour reaction appears to be specific for certain hexoses. Fructose gives about 20 % more colour than glucose, while the saccharides derived from these two hexoses, e.g. sucrose, glycogen,

Table 1. *Colour reaction of various carbohydrates and derivatives in hot sulphuric acid*

One ml. of a solution containing 0·1 mg. of the compounds listed below was heated with 3 ml. of 96 % (w/w) H_2SO_4 at 100° for 6·5 min. The intensity of the pink colour produced was measured at 520 mμ. in a 1 cm. cuvette with a Beckman spectrophotometer and is given below as $E_{520\,m\mu.}$. In the last column of the table, the colour intensity has been expressed as a percentage of that obtained with an equimolar amount of glucose for the compounds 1–5, 13, 14 or, in the case of the di-, tri- and poly-saccharides, 19–23, as a percentage of the colour obtained with an equivalent amount of the constituent monosaccharides.

No.	Compound	Visible colour	$E_{520\,m\mu.}$	Relative colour intensity (%)
1	D-Glucose	Bluish pink	0·160	100
2	L-Glucose	Bluish pink	0·160	100
3	D-Fructose	Bluish pink	0·188	117
4	Sorbose (D and L)	Bluish pink	0·144	90
5	D-Mannose	Yellowish pink	0·045	28
6	D-Galactose	Yellow	—	—
7	DL-Glyceraldehyde	None	—	—
8	Arabinose	Faint yellow	—	—
9	Xylose	Faint yellow	—	—
10	Rhamnose	Faint yellow	—	—
11	Glucoheptose	Yellow brown	—	—
12	Glucoöctose	Yellow brown	—	—
13	Glucose 1-phosphate (NH_4K salt)	Bluish pink	0·091	99
14	Glucose 6-phosphate (Ba salt)	None	0·009	12
15	Glucose 1:6-diphosphate	None	—	—
16	Fructose 1:6-diphosphate	None	—	—
17	Glucuronic acid	Faint yellow	—	—
18	Sorbitol	None	—	—
19	Sucrose	Bluish pink	0·177	107
20	Raffinose	Bluish pink	0·134	101
21	Inulin	Bluish pink	0·201	96
22	Glycogen	Bluish pink	0·178	101
23	Starch	Bluish pink	0·170	96

inulin and starch, give a pink colour of proportionate intensity (Table 1); little pink colour is developed from mannose. Glucose 1-phosphate is readily hydrolysed in acid (cf. Umbreit, Burris & Stauffer, 1945) and gives the same colour intensity as an equivalent amount of glucose. Glucose 6-phosphate, on the other hand, is relatively resistant to acid hydrolysis (cf. Umbreit *et al.* 1945) and does not give any pink colour under these conditions (Table 1), although the characteristic absorption maximum at 318 mμ. develops upon heating with sulphuric acid.

A large number of non-carbohydrate substances which might occur in deproteinized blood filtrates were also heated in 82% sulphuric acid at 100° for 6·5 min.; in no case was a pink colour formed. The effect of these same compounds on the colour reaction given by glucose was investigated; little or no interference was observed in the presence of urea (80 mg./100 ml.) uric acid, creatine, creatinine, ascorbic acid, β-hydroxybutyric acid (all in concentrations of 10 mg./100 ml.), acetone (40 mg./100 ml.), alanine, arginine, aspartic acid, cystine, glutamic acid, glutamine, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, norleucine, phenylalanine, proline, serine, threonine and valine (all in concentrations of 25 mg./100 ml.). The only substances tested which were found to interfere appreciably with the formation of the bluish pink compound from glucose were sodium chloride (cf. Mendel & Bauch, 1926), ergothioneine, glutathione, tryptophan, methionine and tyrosine (Table 2). The concentrations of the last three amino acids in blood are relatively low (Johnson & Bergeim, 1951) so that the amounts found in a 20-fold diluted blood filtrate would not interfere with the colorimetric determination of glucose.

Effect of chloride

Mendel & Bauch (1926) reported that the presence of chloride ion interfered with the production of the pink colour from glucose in heated sulphuric acid. This effect is illustrated in Fig. 6. The formation of the bluish pink compound with an absorption maximum at 520 mμ. is inhibited when the concentration of sodium chloride in the aqueous glucose solution is greater than 2·5 mg./100 ml. The amount of HMF formed, on the other hand, is increased in the presence of chloride. Apparently more glucose is converted into HMF in the presence of chloride, while the intermediary reaction leading to the coloured compound is prevented.

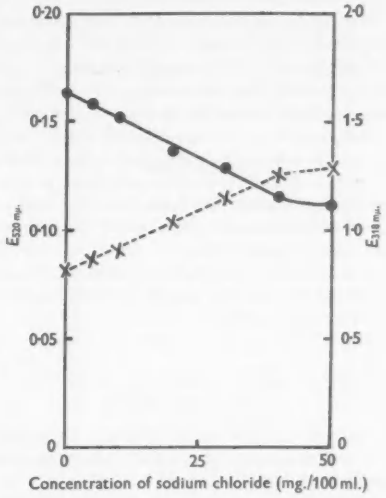

Fig. 6. Glucose solutions (0·01%) containing various concentrations of NaCl were heated with three volumes of 96% (w/w) H_2SO_4 at 100° for 6·5 min. The extinctions were measured at 520 mμ. (●—●) and at 318 mμ. (×– – –×); the figures obtained for the extinction at 318 mμ. are plotted on one-tenth of the scale used for the values at 520 mμ.

Table 2. *Effect of substances found to interfere with the colour reaction*

One ml. of an aqueous solution containing 0·1 mg. glucose and various concentrations of the substances listed below was heated with 3 ml. of H_2SO_4 at 100° for 6·5 min.; the intensity of the pink colour produced was measured at 520 mμ.

Compound added	Amounts occurring in a 20-fold diluted blood filtrate (mg./100 ml.)	Amount added to the glucose solution (mg./100 ml.)	$E_{520\ m\mu.}$
None	—	—	0·160
Sodium chloride	28–31	30·0	0·125
Ergothioneine	0·15–0·6	1·5	0·150
Glutathione	0·8–2·0	5·0	0·119
Tryptophan	0·024–0·05	2·5	0·093
		0·5	0·130
		0·1	0·160
Methionine	0·017–0·035	25·0	0·020
		0·5	0·161
Tyrosine	0·04–0·07	25·0	0·080
		0·5	0·162

Experiments with blood samples

Since TCA was without influence on the production of the pink colour from glucose in heated sulphuric acid, it was selected as deproteinizing agent instead of the unstable 'metaphosphoric acid' used by Mendel & Bauch (1926). By the addition of silver sulphate to the TCA solution the chlorides in the blood sample are precipitated simultaneously with the protein. When 0·1 ml. of blood is pipetted into 1·9 ml. of TCA solution containing 0·1% (w/v) silver sulphate, chlorides are only rarely detectable in the filtrate and never in concentrations higher than 2 mg./100 ml. If desired, 0·2 ml. of blood can be pipetted into 1·8 ml. of the 5% (w/v) TCA solution; however, the concentration of silver sulphate should then be increased to 0·2% (w/v). The excess of silver ions in the filtrate, amounting to less than one-fifth of the amount added, has no effect on the intensity of the pink colour (Table 3).

It appeared that the silver sulphate in the deproteinizing solution also removes the two other interfering substances in the blood, namely glutathione and ergothioneine. Addition of large amounts of glutathione and ergothioneine to normal blood failed to exert any significant influence on the colour reaction (Table 4).

Table 3. *Effect of silver ions on the colour reaction with glucose*

The aqueous solution contained 10 mg. glucose/100 ml. and various concentrations of Ag_2SO_4; the colour reaction was carried out in the usual way.

Concn. of Ag_2SO_4 in the glucose soln. (mg./100 ml.)	$E_{520\ m\mu}$.
0	0·162
10	0·162
50	0·163
100	0·165
200	0·171

Table 4. *Determination of glucose in blood to which glutathione or ergothioneine had been added*

Glutathione or ergothioneine were added to defibrinated human blood, and 0·2 ml. of the blood was deproteinized with 1·8 ml. of 5% (w/v) TCA containing 0·2% (w/v) Ag_2SO_4. One ml. of the clear supernatant fluid obtained after centrifuging was added to 3 ml. of H_2SO_4 and the colour reaction was carried out in the usual way.

Substance added	Amount added to the blood (mg./100 ml.)	$E_{520\ m\mu}$.	Blood glucose found (mg./100 ml.)
None	—	0·153	94
Glutathione	25	0·152	94
Glutathione	50	0·156	96
Ergothioneine	15	0·153	94

Apart from the carbohydrates, other substances present in blood do not give the colour reaction with hot sulphuric acid. No pink colour was formed after the glucose in blood had been removed by fermentation with yeast.

Table 5 shows that the usual anticoagulants do not affect the colorimetric determination of blood sugar. Sodium fluoride in a concentration of 10 mg./ml. appeared to be the most suitable anticoagulant since it prevents glycolysis (Lovatt Evans, 1922) as well as clotting.

Repeated determinations of the blood sugar on the same sample of blood showed that the reproducibility of the results was very satisfactory. The average of eight determinations on one sample of normal human blood was 85·1 mg. glucose/100 ml. (S.D. 1·63 mg./100 ml. or 1·9%). Five determinations on a second sample of normal human blood gave an average of 92·8 mg. glucose/100 ml. (S.D. 1·65 mg./100 ml. or 1·8%). Glucose added to blood could always be recovered completely from the blood filtrates (Table 6).

Table 5. *Determination of the glucose in blood to which anticoagulants had been added*

The anticoagulants listed below were added to defibrinated human blood; 0·1 ml. of the blood was deproteinized with 1·9 ml. of 5% (w/v) TCA containing 0·1% (w/v) Ag_2SO_4. After centrifuging, the colour reaction was carried out in the usual way.

Anti-coagulant added	Amount added to the blood (mg./ml.)	$E_{520\ m\mu}$.	Blood glucose found (mg./100 ml.)
None	—	0·069	85
Sodium oxalate	2	0·069	85
Sodium fluoride	10	0·068	84
Sodium fluoride	25	0·069	85
Heparin	10	0·070	86

Table 6. *Determination of the glucose in blood to which known amounts of glucose had been added*

Known amounts of glucose were dissolved in human blood containing 10 mg. NaF/ml. as anticoagulant; 0·1 ml. of the blood was deproteinized with 1·9 ml. of TCA: Ag_2SO_4 solution, centrifuged, and the colour reaction carried out in the usual way.

Blood sample	Amount of glucose added to the blood (mg./100 ml.)	Blood glucose found (mg./100 ml.)	Glucose recovered (mg./100 ml.)
A	0	65	—
	25	91	26
	50	113	48
	100	160	95
	250	315	250
B	0	79	—
	50	129	50
C	0	71	—
	50	122	51

Table 7. *Comparison of the blood-sugar values determined by the methods of Somogyi and Mendel & Hoogland*

Blood samples were taken from non-fasted experimental animals. Three of the samples were obtained from dogs which had been rendered diabetic by pancreatectomy. The blood-sugar value of each sample was determined by the iodometric method of Somogyi (1945) and by the colorimetric method of Mendel & Hoogland (1950) as described in the text.

Sample no.	Species	Blood sugar (mg./100 ml.) Somogyi	Mendel & Hoogland
1	Rat	120	121
2	Guinea pig	117	115
3	Rabbit	106	100
4	Sheep	39	39
5	Duck	130	130
6	Diabetic dog *A*	284	280
7	Diabetic dog *B*	302	300
8	Diabetic dog *C*	324	312

Finally a comparison was made between the blood-sugar values determined by the present method and the values found on the same samples of blood by the iodometric macro-method of Somogyi (1945). The results obtained with blood from experimental animals are given in Table 7. It can be seen that there is a good agreement between the two methods in almost every case.

DISCUSSION

Most of the accepted methods for the determination of blood sugar, e.g. Folin & Wu (1922) and Hagedorn & Jensen (1923), are based on the reducing properties of glucose. Since blood also contains 'non-sugar' reducing substances, it is difficult to determine its 'true glucose' content with these methods, though more reliable results have been obtained with the techniques developed by Somogyi (1937, 1945, 1952). The colorimetric method described here, on the other hand, does not depend on the reducing power of glucose but on the formation of 5-hydroxymethylfurfural. The colour reaction appears to be specific for the hexoses which occur in blood, i.e. glucose and fructose (Bacon & Bell, 1948). 'Non-sugar' reducing substances are not determined by this method.

Another method which depends on the formation of furfural derivatives from carbohydrates in hot sulphuric acid has been described by Dische (1930, 1947) (cf. Gurin & Hood, 1939, 1941). This method does not appear to be specific for hexoses and in addition requires very pure carbazole as a chromogenic agent. No chromogenic agent is necessary for the formation of the pink colour in the reaction described by Mendel & Bauch (1926). Moreover, the new method is simple, and requires only two reagents which can easily be obtained and which are stable over long periods.

After this paper was completed, Love (1953) reported studies on the spectra which are obtained when carbohydrates are heated with concentrated sulphuric acid. It will be noted that Love (1953) used a higher concentration of sulphuric acid (98 %) and high concentrations of glucose (40–100 mg. 100 ml.) in his investigation of the visible spectra. Both factors lead to an early formation of humic substances which, by their yellow-brown colour, would mask the characteristic bluish pink colour obtained with glucose and fructose under the conditions described in the present paper. Since our method can easily be adapted for measurements with a colour scale (Kemp & Zuidweg, 1952) or with simple colorimeters, it should be suitable for routine determinations of blood glucose or tissue glycogen.

SUMMARY

1. A colorimetric micro-method for the determination of glucose has been described. This method is based on the observation that a bluish pink colour develops when one volume of a dilute solution of glucose is heated with three volumes of 96 % (w/w) sulphuric acid; the intensity of the pink colour is proportional to the concentration of glucose.

2. The glucose is dehydrated in hot sulphuric acid to 5-hydroxymethylfurfural, which reacts with a compound intermediate between glucose and hydroxymethylfurfural to produce the pink colour. For practical purposes, this reaction appears to be specific for glucose, fructose and saccharides containing these two hexoses.

3. The true glucose content of blood can be determined by this simple method after the blood has been deproteinized with a trichloroacetic acid solution containing silver sulphate.

Many of the experiments reported in this paper were done together with Dr P. L. Hoogland in the Banting and Best Department of Medical Research, University of Toronto, Canada; we are greatly indebted to Dr Hoogland for his co-operation. We gratefully acknowledge gifts of glucose 1-phosphate and glucose 6-phosphate from Dr D. M. Needham, F.R.S., Cambridge, of glucoheptose and glucooctose from Prof. H. O. L. Fischer, Berkeley, and of 5-hydroxymethylfurfural from Dr H. C. Beyerman, Amsterdam. We are also much indebted to Dr A. Neuberger, F.R.S., London, for his kind help in finding a suitable brand of sulphuric acid manufactured in Great Britain.

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A Colorimetric Micro-method for the Determination of Glycogen in Tissues

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A new method for the determination of blood sugar has recently been described by Mendel & Hoogland (1950) and Mendel, Kemp & Myers (1954). This method is based on a colour reaction which occurs when a dilute solution of glucose is heated with concentrated sulphuric acid (Mendel & Bauch, 1926). Since glycogen is hydrolysed to glucose in hot sulphuric acid, this reaction can also be used to determine glycogen.

The deproteinizing solution used by Mendel & Hoogland (1950) contains trichloroacetic acid and, in addition, a small amount of silver sulphate to precipitate free chloride and other interfering substances. Although pure glycogen dissolves readily in this deproteinizing solution, only part of the glycogen can be extracted from the tissues with a cold solution of trichloroacetic acid (Willstätter & Rhodewald, 1934; Young, 1937; Bloom, Lewis, Schumpert & Shen, 1951; cf. Przylecki & Majmin, 1934; Mystkowski, 1937). However, it was found in the present investigation that all of the glycogen can be brought into solution by grinding the tissue with trichloroacetic acid solution and then heating the suspension for 15 min. at 100°.

Any glucose present in the tissues will of course be extracted and determined, together with the glycogen by this method. The amount of glucose in the tissues is usually small in comparison with the amount of glycogen present, but under certain experimental conditions it might be desirable to remove the glucose. Procedures will, therefore, be described for the determination of both glycogen and glucose in muscle and liver. Of the glycogen metabolites containing a hexose molecule, only glucose 1-phosphate gives the colour reaction (Mendel *et al.* 1954); however, the concentration of glucose 1-phosphate in tissues (cf. Umbreit, Burris & Stauffer, 1945) is within the limits of error of this determination.

METHODS

Reagents

Deproteinizing solution. Trichloroacetic acid (5 g., A.R.) and Ag_2SO_4 (100 mg., A.R.) are dissolved in water and made up to 100 ml. (cf. Mendel *et al.* 1954). The solution is stored in an amber bottle in the cold.

Sulphuric acid, 96% (w/w, approx. 36N) (cf. Kemp & Zuidweg, 1952; Mendel *et al.* 1954).

Methanol, 80% (v/v).

Procedure

Determination of glycogen plus glucose. Muscle or liver (25–75 mg.) are ground with 5 ml. of the deproteinizing solution in a centrifuge tube; a stainless-steel pestle with longitudinal grooves which fits closely into a cylindrical centrifuge tube has proved useful for this purpose. The fluid level is marked on the centrifuge tube and the tube, covered with a glass cap, is placed in a boiling-water bath for 15 min. Then the tube is cooled in running water, filled up to the mark with deproteinizing solution to compensate for evaporation, and centrifuged at 3000 rev./min. for 5 min. One ml. of the clear supernatant fluid is added to 3 ml. of H_2SO_4 in a wide test tube and mixed by vigorous shaking. The mixture is heated in a boiling-water bath for exactly 6·5 min. and subsequently cooled in running tap water. The intensity of the pink colour produced is measured spectrophotometrically at 520 mμ. and the glycogen concentration read from a standard curve in terms of glucose equivalents. As noted previously (Mendel *et al.* 1954), glycogen gives the same colour intensity as does an equivalent amount of glucose.

The intensity of the pink colour is proportional to the amount of glycogen up to a concentration of 150 μg. glycogen/ml. extract. Should the amount of glycogen in the tissues be so high that the glycogen concentration in the deproteinized extract is greater than 150 μg./ml., as will usually be the case with liver, the extract should be diluted before the colour reaction is carried out. A convenient dilution for normal liver is such that 1 ml. of the deproteinized solution corresponds to 2 mg. of fresh liver tissue.

In view of the uneven distribution of the glycogen in muscle, an alternative procedure may be used in order to obtain better average values. Larger pieces of muscle weighing 200–250 mg. can be extracted with 5 ml. of the deproteinizing solution as described above. However, after heating and restoring the suspension to its original volume, another 5 ml. of the deproteinizing solution should be added to remove any remaining chloride.

Determination of glucose and glycogen separately. The tissue sample is ground with 5 ml. of 80% (v/v) methanol. The suspension is centrifuged and the supernatant fluid containing the glucose is decanted into a calibrated centrifuge tube. Approximately 10 mg. of powdered charcoal are added to this methanol solution; the charcoal does not adsorb any hexose present, but will remove organic substances which would otherwise interfere with the colour reaction. The methanol is now removed completely under reduced pressure while heating the tube in warm water. Deproteinizing solution is added to the residual aqueous solution, still containing the charcoal, to bring the total volume to 5 ml. The suspension is centrifuged and the colour reaction is carried out with 1 ml. of the clear supernatant fluid, as described above. The amount of glucose extracted from the tissue sample in 80% (v/v) methanol can be estimated from the intensity of the pink colour produced.

Since glycogen is insoluble in 80% (v/v) methanol, the glycogen present in the original sample of tissue can be recovered from the precipitated residue remaining after extraction of the glucose with methanol. This tissue residue can be suspended in 5 ml. of deproteinizing fluid, the glycogen extracted by heating at 100° for 15 min. and the amount of glycogen determined as described above.

RESULTS

The results obtained are given in Tables 1 and 2. In order to compare these results with those obtained by the classical methods, the glycogen was also isolated according to Pflüger (cf. Good, Kramer & Somogyi, 1933), dissolved in water, and determined by the colorimetric method (Mendel *et al.* 1954). It can be seen from Tables 1 and 2 that the two methods give very similar values for the glycogen content of tissues. The amount of free glucose in the tissues is small (Table 2) and can usually be neglected when the glycogen content of normal muscle or normal liver is being estimated.

Table 1. *Determination of glycogen in rat gastrocnemius muscle with the new micro-method and with a modification of Pflüger's method*

The glycogen in the gastrocnemius muscle of normally fed rats was extracted with trichloroacetic acid as described in the text, or by a modification of Pflüger's method; in both cases, the amount of glycogen was determined colorimetrically as described by Mendel *et al.* (1954). The glycogen content of the fresh muscle was calculated in terms of glucose equivalents. Mean values are given ±standard error; when only two determinations were made the individual results are given.

	New micro-method			Pflüger's method		
Rat no.	No. of determinations	Wt. of muscle samples (mg.)	Glycogen content (%)	No. of determinations	Wt. of muscle samples (mg.)	Glycogen content (%)
1	5	273–482	0·49±0·017	2	941, 1038	0·54, 0·56
2	5	182–221	0·40±0·009	2	807, 914	0·36, 0·36
3	6	182–215	0·43±0·012	2	979, 1102	0·42, 0·44
4	5	55–79	0·32±0·017	2	1331, 1645	0·35, 0·39
5	7	24–41	0·58±0·015	5	551–926	0·61±0·017

Table 2. *Determination of glycogen and glucose in the liver and gastrocnemius muscle of the rat*

The glycogen and glucose were extracted from the tissues of well-fed animals by both of the procedures described in the text. The carbohydrate extracted from fresh tissue with hot trichloroacetic acid represents glycogen plus glucose; the methanol-soluble fraction represents glucose, while the methanol-insoluble fraction represents glycogen. For purpose of comparison, the glycogen was also isolated from the same tissues by a modification of Pflüger's method. In all cases the amount of carbohydrate was determined colorimetrically as described by Mendel *et al.* (1954) and expressed as glucose equivalents. Mean values are given ±standard error; when only two determinations were made the individual results are given.

		New micro-method				Pflüger's method		
Rat no.	Tissue	No. of determinations	Wt. of tissue samples (mg.)	Glycogen plus glucose (%)	Glucose (%)	Glycogen (%)	No. of determinations	Wt. of tissue samples (mg.)	Glycogen (%)
6	Liver	3	26–73	2·12±0·011	0·44±0·022	1·76±0·05	2	331–396	1·73, 1·61
7	Muscle	3	27–54	0·54±0·011	0·09±0·003	0·45±0·011	3	635–850	0·51±0·017

DISCUSSION

The new micro-method for determination of glycogen in tissues gives reasonably accurate results with 25–75 mg. of muscle or 10 mg. of liver. These results are in good agreement with those obtained by Pflüger's method of extraction; however, the new method has several advantages. In the first place, the glycogen content of a small piece of tissue can be readily determined. Fifteen to twenty determinations can be done on a single rat muscle weighing 2 g.; this would make it possible, for example, to determine the distribution of glycogen within the muscle itself. Similarly, the small amount of liver tissue necessary for the determination would allow the estimation of glycogen in biopsy specimens. Furthermore, the new method is simpler and more rapid than the classical methods; the glycogen content of twenty tissue samples can be determined in about 2 hr.

The procedures described above are concerned only with the determination of the total glycogen content of tissues. It seems possible, however, that two different forms of glycogen may exist in the tissues. As noted previously, only part of the glycogen can be extracted from the tissues with cold trichloroacetic acid, and it is necessary to heat the suspension or to destroy the tissue by alkaline hydrolysis before the remaining glycogen can be brought into solution. The results obtained by Bloom *et al.* (1951) and by Bloom & Knowlton (1953) seem to indicate that the relationship between these two glycogen fractions in muscle can be altered by subjecting the animal to various experimental conditions. The new micro-method for the determination of glycogen could easily be adapted to a study of this problem by carrying out the extraction with deproteinizing solution in two stages, that is, first in the cold and subsequently at 100°.

SUMMARY

1. A simple micro-method for the determination of glycogen in tissues has been described. The tissue is extracted with a solution of trichloroacetic acid at 100° and the glycogen in the extracts is determined, without previous hydrolysis, by the colorimetric method described by Mendel *et al.* (1954).

2. A procedure is described by which the glucose can be extracted from the tissue and determined separately.

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Spectrophotometric Studies of the Reaction of Methaemoglobin with Hydrogen Peroxide

1. THE FORMATION OF METHAEMOGLOBIN-HYDROGEN PEROXIDE

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The unstable red substance formed when hydrogen peroxide is added to an acid methaemoglobin solution (Kobert, 1900) was shown by Keilin & Hartree (1935) and Haurowitz (1935) to be a well-defined compound apparently analogous to the azide, fluoride, cyanide and hydrosulphide compounds of methaemoglobin, with absorption bands at 545 and 589 mμ., and requiring for its formation 1 molecule of hydrogen peroxide for each iron atom of haematin. The compound is unstable, and in the subsequent reaction part of the methaemoglobin is recovered whilst the remainder is lost by oxidative degradation to unidentified products (Keilin & Hartree, 1935).

We have shown that in the reaction between human haemoglobin and hydrogen peroxide in the

presence of an excess of sodium dithionite at pH 8·5, which results ultimately in partial oxidation to choleglobin, a transient intermediate compound is formed, which subsequently reverts, in the main, to haemoglobin by a rather slow reaction (Dalziel & O'Brien, 1951, 1952*a*). The spectral absorption of this transient compound resembles, but is not identical with, that of methaemoglobin-hydrogen peroxide. Lemberg, Legge & Lockwood (1939) postulated the occurrence of a haemoglobin-hydrogen peroxide compound as the precursor of choleglobin in the coupled oxidation of oxyhaemoglobin and ascorbic acid. The same workers remarked that methaemoglobin, as well as haemoglobin and oxyhaemoglobin, yielded choleglobin on reaction with hydrogen peroxide in the absence of reducing agents, and assumed that methaemoglobin was reduced to haemoglobin by the peroxide and thence formed haemoglobin-hydrogen peroxide (Lemberg, Legge & Lockwood, 1941). In seeking to clarify the relationship between the transient compound which occurs in the reaction of haemoglobin, dithionite and hydrogen peroxide, and methaemoglobin-hydrogen peroxide, we have made spectrokinetic studies of the reaction of human methaemoglobin and hydrogen peroxide.

In this paper, experiments with a rapid-reaction apparatus (Dalziel, 1953) are described which show that catalase-free human methaemoglobin is quantitatively converted into methaemoglobin-hydrogen peroxide by an excess of hydrogen peroxide at pH's 6·0 and 8·5. The spectral absorption of the compound in both the visible and Soret regions has been measured, and approximate kinetic and apparent equilibrium data have been obtained. Some observations on the reaction of the compound with sodium dithionite at pH 8·5 are also described. In view of the variability of published spectrophotometric data for methaemoglobin, extinction coefficients for the latter are recorded. Studies of the consecutive reaction of the compound in the presence of excess of peroxide will be described in a second paper. An abstract of the work has been published previously (Dalziel & O'Brien, 1952*b*).

EXPERIMENTAL

Spectrokinetic measurements

The changes of spectral absorption accompanying the reaction were recorded in a form of Hartridge-Roughton rapid-reaction apparatus, consisting essentially of a Beckman D.U. photoelectric spectrophotometer and a constant-flow apparatus. A detailed account of the apparatus has already been published (Dalziel, 1953). The reactants are stored in vessels surrounded by water in a lagged pressure tank, and are driven by gas pressure through capillary jets into a common tube, where they mix completely in 0·01 sec. The reacting mixture flows at a constant rate down the tube and can be analysed spectrophotometrically at selected points. Each of the two jets is connected through a three-way stopcock to two storage vessels, in order that calibration fluids or solvent may be alternately run with the reaction mixture; the analysis is then independent of spectrophotometric constants, and of the path length and optical qualities of the observation tube. If the temperature of the reactants in the pressure tank is within about 1° of room temperature, the temperature of the mixture does not change by more than 0·1° during flow through the leads and observation tube. Slower reactions are followed, after stopping the flow, in an extension to the observation tube surrounded by a jacket through which water at the same temperature as the reactants circulates.

The half-reaction time for methaemoglobin-hydrogen peroxide formation under the conditions of our experiments was found to be of the order of seconds, and most of the reaction could be followed by stopped-flow measurements. In some experiments it was desirable to make measurements in both the visible and the Soret regions of the spectrum with the same pigment concentration, and in these cases the water-jacketed attachment of path length 0·99 cm. was used for the region 495–640 mμ., and the observation tube of path length 0·20 cm. for the region 400–430 mμ. The reactants, methaemoglobin (metHb) and H_2O_2, were made up in buffer solutions; metHb was put in one reactant vessel, buffer in another, and H_2O_2 in the other two, such that appropriate pairs of fluids could be run together. With a particular wavelength setting the procedure was as follows: (1) H_2O_2 and buffer were run together, and the spectrophotometer set to zero extinction in the usual manner; (2) metHb and buffer were run together, and the extinction measured, equal to that of the reaction mixture at the instant of mixing; (3) metHb and H_2O_2 were run together, and the extinction of the flowing mixture measured, corresponding in these experiments to either 0·2 sec. or 0·8 sec. after mixing; flow was then stopped abruptly by closing the exit stopcock at the end of the observation tube, and the time course of the extinction was recorded with a stopwatch; (4) finally, H_2O_2 and buffer were run together again, and the solvent-setting checked. These operations were repeated at other wavelengths. From the series of plots of extinction against time, or spectrokinetic curves, as obtained, the absorption curve of the reaction mixture at any stage of the reaction could be built up.

In preliminary experiments on the overall reaction, the reactants were mixed in an optical cell, and the time course of the extinction was followed at two or three wavelengths by making measurements in rapid succession from about 1 min. after mixing. This was repeated at other wavelengths with fresh reaction mixture.

Kinetic calculations. For an isolated or reversible reaction involving only two coloured substances, reactant R and the stable, quantitative product P, which obey Beer's law, the fractional composition of the reaction mixture at time t after mixing may be calculated from the extinction of the mixture, E_t, at any suitable wavelength by the relation

$$C_t/C_0 = \frac{E_t \sim E_P}{E_R \sim E_P}, \qquad (1)$$

where C_0 = initial concentration of R, C_t = concentration of R at time t, and E_R, E_P = extinctions of R and P at concentration C_0 and the same path length as E_t. Normally, both $(E_t \sim E_P)$ and $(E_R \sim E_P)$ would be measured directly, using calibration fluids (Dalziel, 1953). In the present experi-

ments, since the product is unstable, E_P was estimated from the spectrokinetic curves, and it will be shown that equation (1) gives reasonably accurate results for methaemoglobin-hydrogen peroxide formation in spite of the consecutive reaction. The precision of the analysis depends upon the magnitude of $(E_R \sim E_P)$, the extinction change for complete reaction, and hence upon the wavelength: the maximum error in C_t/C_0 is estimated as $\pm 3\%$ C_0 when $(E_R \sim E_P) > 0{\cdot}1$.

Concentration units and extinction coefficients. It is common practice to express the concentration of a haem pigment solution in terms of the millimolar concentration of haematin, or its equivalent, and to record its spectral absorption in terms of extinction coefficients based on these units. This is appropriate to the present work. The designation of such coefficients as millimolar extinction coefficients, $\epsilon_{mM} = E/cd$, where c is the millimolar concentration of haematin (cf. Lemberg & Legge, 1949) is misleading, however, especially as some workers use this symbol to denote the true millimolar extinction coefficient of a haem pigment (e.g. catalase, Chance & Herbert, 1950). The use of the convenient haematin units would be facilitated by a suitable symbol: in this paper, the pigment concentration will be expressed as m-equiv./l., 'mEq.', where 1 mEq. metHb $\equiv$ 1 mM haematin. The corresponding extinction coefficient, the milliequivalent extinction coefficient, will be denoted by $\epsilon_{mEq.}$.

Reagents

Hydrogen peroxide. The experiments were made with AnalaR '20 vol.' reagent freshly diluted to the required concentration with Clarke & Lubs's 0·05 M phosphate:NaOH, pH 6, or borate:NaOH, pH 8·5. Such solutions were stable for a few hours. The stock reagent was repeatedly analysed by Andrews's KIO_3:As_2O_3 titration. Other experiments with a commercial H_2O_2 sample containing stabilizer gave similar results.

Catalase-free methaemoglobin. Red cells separated from freshly drawn human blood (to which 10 mg. heparin/100 ml. was added) were washed thrice with 0·9% NaCl, and haemolysed in 5 times their volume of distilled water. The haemolysate was diluted with an equal volume of 0·4 M acetate buffer, to give a pH of 5·8, and the precipitate of cell debris immediately removed by centrifuging. Catalase was removed by treating the solution with three or four successive portions of alumina suspension, allowing the mixture to stand with gentle mixing for 5 min. each time, and separating the alumina by centrifuging. The perfectly clear solution of oxyhaemoglobin, having less than 1% of its original catalase content, was oxidized without delay by adding solid $K_3Fe(CN)_6$ to a concentration of about 10–15 mol./haematin mol. In some preparations the oxidation was carried out at pH 5·8, in others the pH was first brought to about 7, and in still others to 8·5. The product was dialysed against tap water, distilled water, and finally borate buffer of pH 8·5, and filtered. The pH at which oxidation was carried out did not significantly affect the spectrum of the product (see below) or the reaction with H_2O_2. But oxidation at pH 8·5 gave a product less stable to extremes of pH; in dilute solution at pH 6 such samples of metHb became turbid quite quickly, and precipitated after some hours.

The concentration of metHb in the preparations was estimated spectrophotometrically by conversion into the cyanide compound, as recommended by Drabkin & Austin (1935). A suitable volume (1 or 2 ml.) was pipetted into a 10 ml. volumetric flask, 0·2 ml. of a solution of KCN containing 0·3 g./100 ml. was added, and the flask made up to the mark. The extinction was measured in 1 cm. cells at 551, 545 and 540 mμ., and the cyanmethaemoglobin concentration calculated from Drabkin's values for $\epsilon_{mEq.}$ at these wavelengths, namely, 11·1, 11·5 and 11·5, respectively.

The metHb preparations were diluted as required in Clarke & Lubs's phosphate (pH 6·0) (cf. Vogel, 1939) or borate (pH 8·5) buffer solutions. In most experiments the initial metHb concentration was about 0·05 mEq., to permit accurate spectrophotometric measurements in the visible and Soret regions with 1 cm. and 0·2 cm. path lengths, respectively. In a few experiments with lower pigment concentrations, down to 0·003 mEq., measurements were confined to the Soret region with 1 cm. path length.

Spectrophotometry of methaemoglobin

The wavelengths of maximum absorption and milliequivalent extinction coefficients for two typical metHb preparations are given in Table 1. These samples were made from the same catalase-free oxyhaemoglobin solution by oxidation at pH's 5·8 and 8·5. The complete absorption curves were measured at pH 6·0 (0·05 M-KH_2PO_4:NaOH) and pH 10·0 (0·05 M-H_3BO_3:KCl:NaOH). Analysis by the micro-Kjeldahl method gave 17·3 and 17·5% for the N content of the samples, assuming 1 mEq. metHb = 16·7 g./l.; these figures and the extinction coefficients are based on Drabkin's data for cyanmethaemoglobin referred to previously.

Table 1. *Absorption maxima and milliequivalent extinction coefficients, $\epsilon_{mEq.}$, of methaemoglobin*

$\epsilon_{mEq.} = 1/cd \log_{10} I_0/I$; c = m-equiv. metHb/l. = m-mol. haematin/l.; d = cm.; N.B.W. = nominal band width. MetHb solutions prepared from alumina-treated haemolysates of washed human red cells, by oxidation with $K_3Fe(CN)_6$, and dialysis. Diluted for measurements with Clarke & Lubs's 0·05 M phosphate (pH 6·0) or borate (pH 10·0).

	$\epsilon_{mEq.}$							
	pH 6·0 Absorption maxima (mμ.)				pH 10·0 Absorption maxima (mμ.)			
	275	405	500	630	275	413	540	576
	N.B.W. (mμ.)							
pH of oxidation	2·8	1·3	1·5	6·2	2·8	1·4	1·8	2·4
5·8	29·6	167	9·3	4·0	32·2	107	10·7	9·6
8·5	29·8	164	9·3	4·0	32·2	106	10·6	9·5

RESULTS

Preliminary experiments in which metHb and H_2O_2 were mixed in optical cells, to give a pigment concentration of about 0·05 mEq. and molar ratios, R, of peroxide to haematin of from 4 to 90, showed the formation of a compound at pH's 6·0 and 8·5 with an absorption spectrum in the visible region similar to that recorded by Haurowitz (1935) and Keilin & Hartree (1951). The compound was unstable, but the subsequent changes of light absorption were relatively slow.

Spectrokinetic measurements

The spectral changes accompanying the early stages of the reaction were studied quantitatively in the rapid reaction apparatus by recording the time course of the extinction at appropriate wavelengths.

In Expt. 1 with 0·049 mEq. metHb and $R = 40$, at pH 6·0, observations were made at the wavelengths: 402, 405, 408, 415, 418, 422, 495, 500, 515, 540, 545, 550, 560, 576, 580, 590, 610, 615, 620, 630, 640 mμ. Some of the spectrokinetic curves are shown in Fig. 1, and the absorption spectra of the reaction mixture at 0·2 or 0·8, 3, 15 and 300 sec. after mixing in Fig. 2. Up to 10–15 sec., the spectrokinetic curves are smooth and continuous, consistent with the transformation of metHb to a single product characterized by the integrated spectrum after 15 sec., with points of approximately constant intensity at 414, 517 and 614 mμ. There is no indication of the formation in appreciable concentration of any spectroscopically distinct intermediate. At about 10–15 sec., the spectrokinetic curves show discontinuities: at 415–422, 540–560 and 620–640 mμ., dE/dt changes sign; at 495 and 500 mμ., a constant value of E succeeds the initial fall; at 615 mμ., E begins to increase and at 402–408 mμ. inflexions are apparent. These changes are attributed to consecutive reactions: (i) the formation of a compound with absorption maxima at 416–418 and 545 mμ. (Fig. 2), followed by (ii) slow destruction of the compound accompanied by decreasing absorption in the whole Soret region and near 545 mμ., and increasing absorption in the region 590–640 mμ.

In a similar experiment with $R = 3$ and the same pigment concentration, again at pH 6·0, the results indicated that the reaction was incomplete, but otherwise differed only in rate from that with the higher peroxide concentration. Up to 2 min., the spectral absorption of the reacting mixture showed isosbestic points in about the same positions as before, and the spectrum at 2 min. was consistent with a mixture of metHb and methaemoglobin-hydrogen peroxide. The consecutive reaction in the period 2–5 min. was also slower. As in Expt. 1 ($R = 40$), a decline of the maximum at 545 mμ. was accompanied by increasing absorption in the region 610–640 mμ., but the uniform decrease of absorption in the Soret region was not reproduced with this lower peroxide concentration; in fact, the absorption at 402–408 mμ. remained constant from 2 to 5 min., and then began to increase again, suggesting reversion to metHb.

Less comprehensive experiments were made with R values of 0·7, 7, 15, 28 and 110 (Expts. 3–7) and the same pigment concentration of 0·049 mEq.

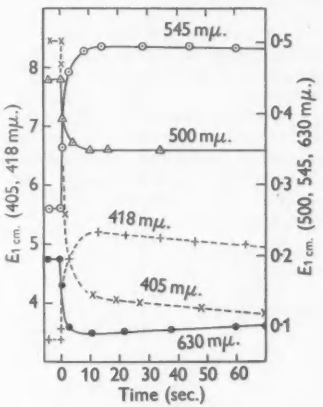

Fig. 1.

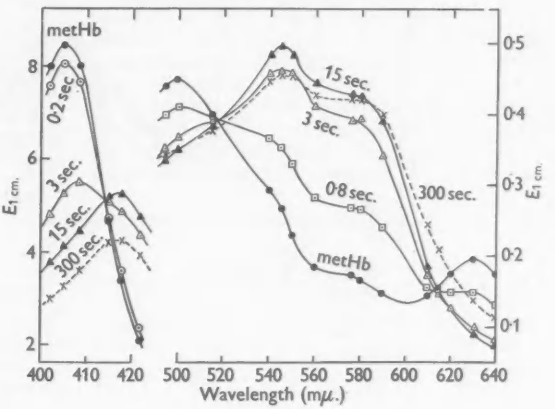

Fig. 2.

Fig. 1. Changes of extinction with time for a reacting mixture of 0·049 mEq. metHb and 2·0 mM-H_2O_2, pH 6·0 (Expt. 1).

Fig. 2. Spectral absorption of a reacting mixture of 0·049 mEq. metHb and 2·0 mM-H_2O_2, pH 6·0, at intervals from 0 to 300 sec. after mixing (Expt. 1).

Observations were restricted to the wavelengths of the absorption maxima of the compound, 418 and 545 mμ., and of metHb, 405, 500 and 630 mμ. The spectrokinetic curves for 418 and 500 mμ. (Fig. 3) show how the rate and the extent of the reaction increase with R. Once again the coincidence in time of the breaks in the curves for different wavelengths is consistent with essentially separate consecutive reactions. The curves for 418 mμ. show that the rate of destruction of the compound also increases with R, but it is evident from the slopes that at each peroxide concentration it is too slow to significantly depress the maximum extinction value. At 500 mμ., the consecutive reaction is not accompanied by significant change of extinction, and measurements at this wavelength should yield reliable estimates of the proportion of free metHb. Both sets of curves show that with $R>40$, the metHb is rapidly and almost completely converted into the compound, whilst with lower peroxide concentrations free metHb persists.

Fig. 4 shows the changes in light absorption of a reaction mixture at pH 8·5, with 0·046 mEq. metHb and $R=60$ (Expt. 8). The spectrokinetic curves at eighteen wavelengths, from which the curves of Fig. 5 were constructed, are smooth and continuous up to 1 min. after mixing, and the absorption spectrum of the mixture then agrees

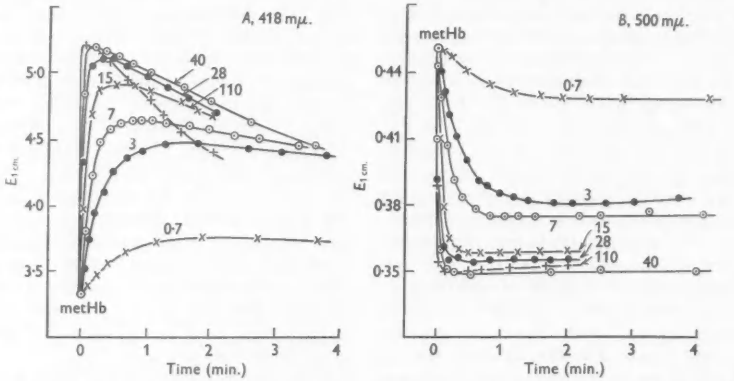

Fig. 3. Time course of the extinction at 418 mμ. (*A*) and 500 mμ. (*B*) for reacting mixtures of 0·049 mEq. metHb and various amounts of H_2O_2, indicated as the molar ratio H_2O_2/haematin; pH 6·0 (Expts. 1–7).

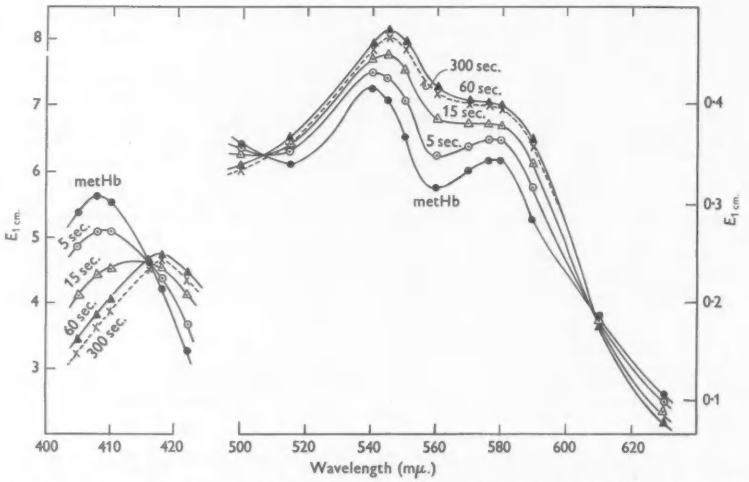

Fig. 4. Spectral absorptions of a reacting mixture of 0·046 mEq. metHb and 2·8 mM-H_2O_2, pH 8·5, at intervals from 0 to 300 sec. after mixing (Expt. 8).

satisfactorily with the 10 sec. spectrum of Expt. 1 at pH 6·0 (Fig. 1). The destruction of the compound in the period 1–5 min. is slower than at pH 6·0, and is accompanied by decreasing or constant extinction at all wavelengths, including 610 and 630 mμ. Less detailed experiments with $R = 26$ and the $R = 130$ and the same pigment concentration showed that the rate of compound formation increased with R but was considerably smaller than with the corresponding peroxide concentrations at pH 6·0, and did not vary significantly in extent over this range of R values.

Experiments with lower pigment concentrations and higher R values at pH's 6·0, 6·9 and 8·5, in which observations were made at ten wavelengths from 400 to 430 mμ., gave similar results, and confirmed the location of an absorption maximum at 416–418 mμ. due to methaemoglobin-hydrogen peroxide. The peroxide used in these experiments (*a–e*) contained stabilizer.

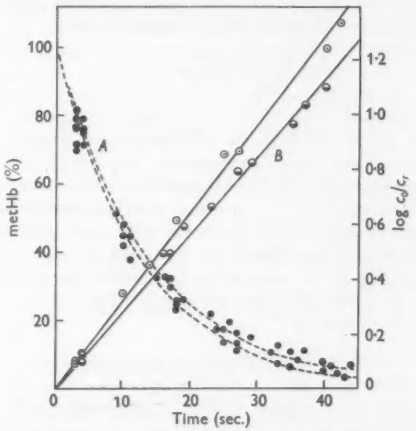

Fig. 5. Rate of reaction of metHb + H_2O_2→compound at pH 8·5 (Expt. 8). *A*: ●, % metHb calculated from extinction measurements at ten wavelengths; the broken lines are theoretical first-order curves for half-reaction times of 9 and 10 sec. *B*: log C_0/C_t, where C_0 = initial metHb concentration, C_t = metHb concentration at time t, calculated from the best extinction measurements, ◒, 405, 408 and 410 mμ.; ⊙, 418 and 422 mμ.

Quantitative interpretation of the spectrokinetic data

The spectrokinetic observations suggest that for the purposes of approximate quantitative treatment the formation of methaemoglobin-hydrogen peroxide in the presence of excess of peroxide ($R > 40$ at pH 6·0, > 20 at pH 8·5) may be regarded as an isolated reaction. Although the compound is unstable, the extinction changes accompanying its subsequent reaction are at most wavelengths, and over a range of H_2O_2 concentration, slow relative to those accompanying its formation. It will be assumed that when E 418 (i.e. E at 418 mμ.) and E 545 reach a maximum the metHb has been completely converted into methaemoglobin-hydrogen peroxide, and molar extinction coefficients for the latter will be estimated. The concentrations of free and combined metHb at any stage during the initial reaction may be calculated approximately from the spectrokinetic curves at any wavelength by equation (1).

Extinction coefficients for methaemoglobin-hydrogen peroxide. In Table 2, extinction ratios $\hat{E}418/E_0408$ and $\hat{E}545/E_0545$ are shown for experiments at pH 8·5, and $\hat{E}418/E_0405$ and $\hat{E}545/E_0500$ for experiments at pH 6·0. For each experiment the E_0 values are measurements on the metHb calibration fluid and the $\hat{E}$ values are the maximum extinctions reached by the reaction mixture. The ratios are constant at a given pH, independent of R, the initial metHb concentration, and the path length, supporting the view that they are reasonably accurate absorption ratios compound/metHb. Independent estimates of $\epsilon_{mEq.}$ were made for each experiment from $\hat{E}418$ and $\hat{E}545$, the optical path length, and the concentration of the compound = initial metHb concentration, the latter being calculated from the

Table 2. *Spectrophotometric constants for methaemoglobin-hydrogen peroxide*

		Composition of reaction mixture		Absorption ratios. Compound, $E\lambda_1$/metHb, $E\lambda_2$				$\epsilon_{mEq.}$ $\lambda_{max.}$ (mμ.)	
pH	Expt.	MetHb concentration (mEq.)	Molar ratio H_2O_2/haematin (R)	418/405	418/408	545/500	545/545	418	545
8·5	8	0·046	60	—	0·84	—	1·17	103	10·4
	9	0·048	26	—	0·86	—	1·18	109	10·7
	10	0·043	130	—	0·83	—	1·18	108	10·9
	c	0·0030	600	—	0·86	—	—	104	—
	d	0·0032	550	—	0·85	—	—	101	—
	e	0·016	110	—	0·86	—	—	102	—
6·9	*b*	0·0030	600	—	—	—	—	107	—
6·0	1	0·049	40	0·61	—	1·10	—	106	10·2
	7	0·049	110	0·62	—	1·12	—	106	10·4
	a	0·0030	600	0·63	—	—	—	104	—

concentration of the metHb reagent and the measured relative flow rates of the reactants in the kinetic apparatus. In view of the combined uncertainties of these quantities, the agreement between the values so obtained (Table 2) is satisfactory, and the spectral absorption of methaemoglobin-hydrogen peroxide is evidently not significantly affected by pH in the range 6·0–8·5.

Kinetics of methaemoglobin-hydrogen peroxide formation. For the experiments with high peroxide concentrations listed in Table 2, the spectrokinetic data at any wavelength may be transposed into approximate kinetic data by equation (1) in the form:

$$\% \text{ metHb} = 100\,\frac{C_t}{C_0} = 100\,\frac{(E_t \sim E_x)}{(E_0 \sim E_x)}, \qquad (1a)$$

where C_0 = initial metHb concentration, C_t = metHb concentration at time t, E_0 = extinction of metHb calibration fluid, E_t = extinction of reaction mixture at time t ($<x$), and E_x = extinction of reaction mixture at time x such that $E_x 418 = E 418$. The kinetic curves so obtained will deviate from the true kinetic curve for the reaction to varying extents at different wavelengths, depending upon the extent to which, as a result of the consecutive reaction of the compound, E_x deviates from the true extinction at complete reaction. Moreover, the deviations may be in either sense, depending upon whether the decomposition enhances or diminishes the extinction change associated with compound formation. A reasonable estimate of the true kinetic curve might therefore be obtained, in spite of the instability of the product, as a mean of the results for wavelengths subject to opposite errors.

Fig. 5 shows the plot of % metHb versus time for Expt. 8, pH 8·5, calculated by equation (1*a*) from the spectrokinetic measurements at the ten wavelengths for which $(E_0 \sim E_x) > 0{\cdot}07$. The points fall, as closely as can be expected, on a logarithmic curve; the broken lines are the theoretical first-order curves for half-reaction times, $t_{0{\cdot}5}$, of 9 and 10 sec.

Fig. 5 also shows the plot of $\log C_0/C_t$ versus time for the most precise data of the same experiment, namely the measurements at 405, 408, 410, 418 and 422 mμ. The points for 418 and 422 mμ. fall on one straight line through the origin, and those for 405, 408 and 410 mμ. on another, in accordance with the form of equation (1*a*) and the expected deviations discussed above: at 405, 408 and 410 mμ. destruction of methaemoglobin-hydrogen peroxide enhances the extinction change associated with its formation, whilst at 418 and 422 mμ. the reverse is true. The mean straight line through the origin should be a good approximation to the true plot, and gives $t_{0{\cdot}5} = 10$ sec.

The data of other experiments at pH 8·5 with excess of peroxide yielded similar results, and therefore the initial course of the reaction in each experiment is satisfactorily described by

$$\frac{\text{d [compound]}}{\text{d}t} = k\,[\text{metHb}]. \qquad (2)$$

Estimates of $t_{0{\cdot}5}$ for the various experiments are given in Table 3. The data for Expts. *c–e* show that with a given excess of peroxide, $t_{0{\cdot}5}$ is independent of the initial metHb concentration. It can be seen that with constant metHb concentration the reaction rate increases with the peroxide concentration (Expts. 8–10), and the function $t_{0{\cdot}5} \times [H_2O_2]$ is reasonably constant, at a given temperature. The kinetics at pH 8·5 may therefore be described by

$$\frac{\text{d [compound]}}{\text{d}t} = k_2\,[H_2O_2]\,[\text{metHb}]. \qquad (3)$$

Values for the second-order constant, k_2, are given in Table 3, and increase significantly with increase of temperature.

Table 3 also shows kinetic data for the few experiments with excess of peroxide at pH's 6·0 and 6·9. The values for $t_{0{\cdot}5}$ are less reliable, especially at pH 6·0, because the reaction is faster and only two or three kinetic measurements were made at each

Table 3. *Kinetic data for methaemoglobin-hydrogen peroxide formation*

pH	Temp. (°)	Expt.	Reactant concentrations: MetHb (mEq.)	Reactant concentrations: H_2O_2 (mM)	Half-reaction time, $t_{0{\cdot}5}$ (sec.)	Sp. reaction rate $k_2 = 0{\cdot}69\,[H_2O_2]/t_{0{\cdot}5}$ (mM^{-1} $sec.^{-1}$)
8·5	23	8	0·046	2·8	10	0·025
		9	0·048	1·25	18	0·030
		10	0·043	5·6	4·7	0·027
	17·4	*c*	0·0030	1·8	20	0·019
		d	0·0032	1·8	22	0·018
		e	0·016	1·8	22	0·018
6·9	17·4	*b*	0·0030	1·8	5·0	0·077
6·0	23	1	0·049	2·0	1·15	0·30
		7	0·049	5·4	0·55	0·23
	17·4	*a*	0·0030	1·8	1·8	0·22

wavelength. Nevertheless, the combined data for fifteen wavelengths were consistent, in each experiment, with equation (2). The results of the three experiments at pH 6·0, which cover quite a wide range of reactant concentrations, also conform reasonably well with equation (3), as shown by the constancy of k_2. The specific reaction rate increases markedly with increase of acidity.

The reaction with low peroxide concentration, pH 6·0. The effect of peroxide concentration on the extent of methaemoglobin-hydrogen peroxide formation at pH 6·0, with initial metHb concentrations of 0·049 and 0·163 mEq., was studied in optical cells by extinction measurements at 500 mμ., where the effect of the consecutive reaction on the light absorption is negligible. With the higher pigment concentration, 60 % compound formation was obtained with $R = 0{\cdot}5$; this, and the kinetic data with excess of peroxide, is in accordance with the requirement of 1 mol. hydrogen peroxide per haematin group, deduced by Keilin & Hartree (1935) from spectroscopic measurements at three times this pigment concentration.

The maximum concentration of methaemoglobin-hydrogen peroxide was attained rapidly under these conditions, and in spite of the presence of traces of catalase, it seemed worth while to test the conformity of the data with the equation

$$\text{metHb} + \text{H}_2\text{O}_2 \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{compound}$$

(where metHb represents one haematin equivalent) on the assumption that the peroxide concentration was the initial concentration less that used in compound formation. The results are shown in Fig. 6 as a plot of log [compound]/[metHb] against log $[H_2O_2]$. The points do not deviate significantly from a straight line of unit slope, and give an apparent $pK = 4{\cdot}4$ at 23°.

A further test of the formulation of the reaction as a simple equilibrium is the ability of the equation

$$\frac{d\,[\text{compound}]}{dt} = k_2\,[\text{metHb}]\,[H_2O_2] - k_1\,[\text{compound}] \qquad (4)$$

to describe the kinetics of the reaction with low peroxide concentrations. If a = initial peroxide concentration in mol./l., b = initial metHb concentration in equiv./l., and x = compound concentration in equiv./l., this equation leads to the relation

$$x_1 e^{k_1 t_1} - x_2 e^{k_1 t_2} = k_2 \int_{t_1}^{t_2} e^{-k_1 t}\,(b-x)\,(a-x)\,dt.$$

The integral can be evaluated graphically for chosen time intervals from experimental values of x and t, and if k_1 is known independently, values of k_2 may be calculated for the several intervals (Hartridge & Roughton, 1925).

The experimental values of $K = 4 \times 10^{-5}$ M and $k_2 = 270\ \text{M}^{-1}\ \text{sec.}^{-1}$ give an approximate value for $k_1 = K \times k_2 = 0{\cdot}011\ \text{sec.}^{-1}$ at 23° and pH 6·0. From the data of Expt. 2, $R = 3$, in which the maximum concentration of methaemoglobin-hydrogen peroxide (68 %) was reached in about 100 sec., values of k_2 were calculated from the concentration changes in successive time intervals for several assumed values of k_1 around 0·01 sec.^{-1}, and are plotted against the mean times in Fig. 7. It can be seen that for the early stages of the reaction, $k_2 = 240\ \text{M}^{-1}\ \text{sec.}^{-1}$, in satisfactory agreement with the value obtained with higher peroxide concentrations, and that if $k_1 = 0{\cdot}010$–$0{\cdot}015$, reasonably constant values of k_2 are obtained throughout the reaction. Similar results were obtained with the data of Expt. 3, $R = 0{\cdot}7$. Within the rather large experimental error, therefore, equation (4) apparently represents the kinetics of incomplete methaemoglobin-hydrogen peroxide formation at low peroxide concentrations.

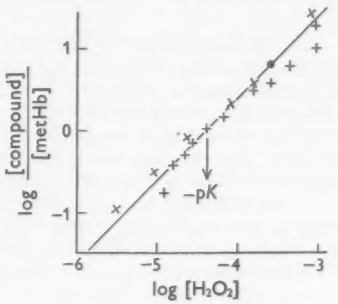

Fig. 6. Effect of H_2O_2 concentration on the extent of compound formation. ×, 0·163 mEq. metHb; +, 0·049 mEq. metHb.

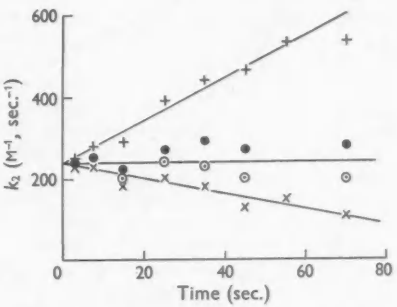

Fig. 7. Test of fit of d [compound]/d$t = k_2$ [metHb] $[H_2O_2]$ $- k_1$ [compound] to the kinetics of incomplete compound formation; Expt. 2, 0·049 mEq. metHb, 0·15 mM-H_2O_2. Values of k_2, calculated from the measured concentration changes in successive time intervals for several assumed values of k_1, are plotted against the mean times. The k_1 values are, ×, 0·005; ⊙, 0·010; ●, 0·015; +, 0·030 sec.^{-1}.

Reaction of methaemoglobin-hydrogen peroxide with sodium dithionite at pH 8·5

Solutions of methaemoglobin-hydrogen peroxide and of dithionite ($Na_2S_2O_4$), buffered at pH 6·0, were mixed in the kinetic apparatus, and spectrokinetic curves were recorded by the stopped-flow method at several wavelengths.

Because of the instability of methaemoglobin-hydrogen peroxide, a fresh sample was prepared for each wavelength and each set of concentration conditions studied, and reaction was brought about within a few minutes. Samples of the reaction mixture were also collected from the apparatus and stored under paraffin; subsequently, the absorption spectra of the products and of denatured globin carboxyhaemochromogens prepared from them by the method of Lemberg *et al.* (1941), were recorded. In most of the experiments the initial pigment concentration in the reaction mixture was 0·02 mEq.; initial H_2O_2 concentrations of 1, 3 and 7 mM. were used with 0·8% dithionite; and with an initial H_2O_2 concentration of 1 mM., dithionite concentrations of 0·05, 0·1, 0·2, 0·4, 0·8, and 2·0% (w/v) were used.

The absorption spectra of the products, and of carboxyhaemochromogen derivatives, were consistent with mixtures of haemoglobin and choleglobin in varying amounts, and showed that protohaematin was destroyed to an extent which increased with the peroxide concentration and diminished with increase of the dithionite concentration. Correspondingly, the yield of choleglobin was greatest with high H_2O_2 concentrations but tended to increase, rather than diminish, with increase of dithionite concentration, so that the loss of protohaematin was not entirely accounted for by choleglobin formation.

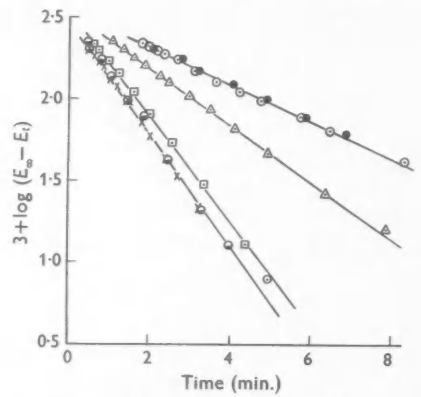

Fig. 8. Kinetics of reduction of 0·02 mEq. methaemoglobin-hydrogen peroxide solutions, containing 1 mM-H_2O_2, by $Na_2S_2O_4$ at various concentrations: ⊙, 0·1; △, 0·2; ⊡, 0·4; ◒, 0·8; and, ×, 2·0 g./100 ml. $Na_2S_2O_4$, pH 8·5, 18°. $(E_\infty - E_t)$ may be assumed to be proportional to the methaemoglobin-hydrogen peroxide concentration at time t after adding $Na_2S_2O_4$.

The spectrokinetic curves, recorded at the wavelengths of the absorption maxima of methaemoglobin-hydrogen peroxide, haemoglobin and choleglobin, namely 418, 430, 545, 555 and 630 mμ., and also at 580 and 600 mμ., showed that at least two reactions occurred. At the haemoglobin maxima, 430 and 555 mμ., the extinction at first decreased for about 30 sec., and then increased slowly in accordance with haemoglobin formation. The initial fall of extinction increased both in magnitude and duration with increase of the peroxide concentration and decreased with increase of the dithionite concentration. The rate of the subsequent increase of extinction at 430 mμ. increased with the dithionite concentration up to 0·8%. Further increase of dithionite concentration did not affect the rate of haemoglobin formation significantly, nor did the peroxide concentration. The effect of dithionite concentration is shown in Fig. 8 as a plot of log $(E_\infty - E_t)$ for 430 mμ. against time, where E_∞ = extinction of the final products and E_t = extinction of the mixture at time t after mixing. The graphs are linear for concentrations of dithionite greater than 0·1%. If it is assumed that after E430 begins to increase the reaction consists solely of the conversion of methaemoglobin-hydrogen peroxide into haemoglobin, then $(E_\infty - E_t)$ is proportional to the concentration of the former substance. The half-time for the reaction, calculated from the slopes, decreases from 152 sec. with 0·1% dithionite to 50 sec. with 0·8–2·0% dithionite at about 18°.

There was no evidence, at the few wavelengths studied, for the formation of any intermediate in the reaction.

DISCUSSION

The time course of the spectral absorption from 0·2 sec. after mixing human methaemoglobin and hydrogen peroxide suggests that at both pH's 6·0 and 8·5 the initial reaction is the formation of a single compound by an essentially isolated and quantitative reaction. With an excess of peroxide, this reaction goes rapidly to completion before significant loss by other reactions occurs; the absorption spectrum of the unstable product was quantitatively reproduced with a range of methaemoglobin and excess of peroxide concentrations, and was identical at pH's 6·0 and 8·5. In addition to a maximum at 545 mμ., $\epsilon_{mEq.} = 10{\cdot}5$, and a pronounced shoulder at 570–580 mμ., recorded previously for methaemoglobin-hydrogen peroxide by Haurowitz (1935) and by Keilin & Hartree (1951) the compound has a sharp peak at 418 mμ., $\epsilon_{mEq.} = 105$. With low peroxide concentrations at pH 6·0, the spectrokinetic curves during the initial stages of the reaction were consistent with a partial

conversion of methaemoglobin into methaemoglobin-hydrogen peroxide, and this reaction appeared to reach an equilibrium before the consecutive reaction of the unstable compound occurred to a significant extent.

Metmyoglobin forms an apparently analogous hydrogen peroxide compound (Keilin & Hartree, 1951). George & Irvine (1952*a*), working with horse metmyoglobin, concluded that only at pH 8·0–9·0 is metmyoglobin quantitatively converted into the compound by hydrogen peroxide. Although the absorption spectrum of the compound prepared in this pH range was unaffected by subsequent change of pH, the absorption spectra of the products of the reaction at pH's 5·8 and 6·8, and of haemochromogen derivatives, showed that metmyoglobin-hydrogen peroxide was not the only product, and it was concluded that simultaneous side reactions were brought about by a transient oxidizing entity produced during compound formation. The time course of the spectral changes accompanying the reaction was apparently not studied, and the time after mixing at which the absorption curves were recorded is not stated. The molar ratio of hydrogen peroxide to haematin in these experiments was much less than in ours, and in later work George & Irvine (1953) found that with excess of peroxide the simultaneous side reactions were eliminated. The products of the side reactions apparently resembled those formed from methaemoglobin-hydrogen peroxide in the presence of excess of peroxide at pH 6·0 by a slow consecutive reaction (Dalziel & O'Brien, 1954).

The absorption spectra of the hydrogen peroxide compounds of methaemoglobin and metmyoglobin are quite analogous. The metmyoglobin compound has absorption maxima at 423 and 549 mμ. (George & Irvine, 1951), 4–5 mμ. nearer to the red than those of the methaemoglobin compound, which is the usual difference exhibited by corresponding myoglobin and haemoglobin derivatives; the millimolar extinction coefficients, 105 and 9·8, respectively, are in good agreement with those of methaemoglobin hydrogen peroxide. It is of interest to compare the Soret spectra of these compounds with those of the secondary hydrogen peroxide compounds of catalase and peroxidase. The shift of the maximum from 405 mμ. in acid methaemoglobin to 418 mμ. in methaemoglobin-hydrogen peroxide may be compared with similar changes from 402 mμ. in horse-radish peroxidase to 417 mμ. in compounds II and III (Chance, 1949*a*, 1952) and from 405 mμ. in catalase to 410 mμ. in the erythrocyte catalase compound II (Chance, 1950) and to 425 mμ. in the bacterial catalase compound II (Chance & Herbert, 1950). The methaemoglobin and metmyoglobin compounds resemble the catalase compounds II and III in having less intense Soret bands than the parent haemoprotein; this is not true of the peroxidase compounds II and III (Chance, 1952; George, 1953). On the basis of light absorption in the visible region, the methaemoglobin and metmyoglobin compounds have been classified with the type III compounds of hydroperoxidases (Keilin & Hartree, 1951). There was no evidence in our experiments for the formation of more than one compound of methaemoglobin with hydrogen peroxide, the spectrokinetic curve at each wavelength studied passing without significant discontinuity from the methaemoglobin value through the earliest point at 0·2 or 0·8 sec. after mixing (Fig. 1).

The consecutive reaction of methaemoglobin-hydrogen peroxide was slow enough to permit reasonably reproducible kinetic data for the formation of the compound to be deduced from the spectrokinetic curves at a number of wavelengths. With high peroxide concentrations at pH's 6·0 and 8·5 the data are satisfactorily described by the equation for a second-order reaction. The approximate value of 220 M^{-1} sec.$^{-1}$ for the velocity constant at pH 6·0 and 17° may be compared with 170 M^{-1} sec.$^{-1}$ for acid metmyoglobin (horse heart) at 18° (George & Irvine, 1953). The specific reaction rate decreases with increase of pH, and inspection of the limited data of Table 3 suggests that, even if alkaline methaemoglobin does not react with hydrogen peroxide at an appreciable rate, the decreases of the rate constant from pH 6·0 to 6·9 and to 8·5 are too great to be attributed to the acidic dissociation at the iron atom, for which $pK = 8{\cdot}1$. If hydrogen ions do not participate in the reaction directly, some other haem-linked group or groups with lower pK values are apparently operative. Now that the general character of the initial reaction and the effect of the consecutive reaction on the spectrum has been made clear, it should be possible to obtain more accurate kinetic data by suitable choice of wavelength and reactant concentrations.

The freshly formed compound can be converted quantitatively into denatured globin carboxy-haemochromogen (Dalziel & O'Brien, 1954) and during the destruction of the compound, whilst the hydrogen peroxide is destroyed, part of the pigment undergoes degradation and part reverts to methaemoglobin (Keilin & Hartree, 1935; Dalziel & O'Brien, 1954). The view that the compound is a reversible complex, analogous to the azide, fluoride, hydrosulphide and cyanide derivatives of methaemoglobin (Keilin & Hartree, 1951) would appear to receive some support from equilibrium and kinetic measurements with low peroxide concentrations at pH 6·0. The results, admittedly of uncertain significance, are consistent with the formulation of the reaction as a simple reversible system

$$\text{metHb} + H_2O_2 \rightleftharpoons \text{compound}$$

(where metHb represents one haematin equivalent) for which $pK = 4{\cdot}4$ at pH 6·0. Our experiments, however, provide no direct evidence of the structure of the compound. The most obvious formulation of the reaction, accounting for its apparent reversibility, would be the replacement of the water molecule on the iron atom of acid methaemoglobin (Coryell, Stitt & Pauling, 1937; Keilin & Hartree, 1949) by a hydrogen peroxide molecule or ion, OOH^-; in view of the greater acidic properties of hydrogen peroxide compared with water, and the fact that the spectrum of the compound is of the alkaline methaemoglobin type and unaffected by pH in the range 6·0–8·5, the hydroperoxide structure, analogous to that proposed by Chance (1949*b*, 1951) for the peroxidase and catalase compounds, would seem to be the more probable. George & Irvine (1951, 1952*a*, *b*) have shown, however, that such structures are unsatisfactory for metmyoglobin-hydrogen peroxide, as well as for the compound II of peroxidase (George, 1952), since these compounds are reduced to the original ferric haemoprotein by ferrocyanide in a one-equivalent step. Moreover, an apparently identical metmyoglobin complex can be formed by the action of oxidizing agents other than hydrogen peroxide (George & Irvine, 1953). George (1952) has suggested, amongst other possibilities, that the compounds contain quadrivalent iron, perhaps as the ferryl derivative, FeO. Magnetic-susceptibility measurements on metmyoglobin-hydrogen peroxide and the peroxidase compound II indicate the presence of two unpaired electrons, and therefore essentially covalent bonding of the iron atom (Theorell, Ehrenberg & Chance, 1952; Theorell & Ehrenberg, 1952). George & Irvine (1952*a*) also found the effect of absolute concentration on the extent of metmyoglobin-hydrogen peroxide formation to be much smaller than would be expected for a reversible equilibrium, and formulated the reaction as an irreversible one of the type

$$\text{metHb} + H_2O_2 = \text{compound} + X,$$

where X is a transient entity possessing one oxidizing equivalent.

There is obviously as yet insufficient experimental data on which to base a comparison of the methaemoglobin and metmyoglobin-hydrogen peroxide compounds; in particular, studies of the mechanism of the side reactions which occur during the formation of the metmyoglobin compound, and of its subsequent reaction in presence of excess of peroxide, are required on the one hand, and a determination of the effective oxidation number of the methaemoglobin compound on the other, as well as more detailed studies of the effect of concentration on the extent of compound formation in both acid and alkaline solution. Magnetic-susceptibility measurements on the methaemoglobin compound have not yet been reported, but are said to be under way (Theorell *et al.* 1952). The absorption spectra of the two compounds are characteristic of analogous haemoglobin and myoglobin compounds, and it seems likely that the two compounds have the same structure, or at any rate that such differences as there may be are common to other pairs of corresponding derivatives. But it is not improbable that the two compounds should react differently, for example, with excess of hydrogen peroxide, and it may be that the apparent reversibility of the methaemoglobin compound and its slow irreversible destruction (Dalziel & O'Brien, 1954) involve intramolecular reactions between the four haems not possible for the metmyoglobin compound. It must be emphasized, however, that the human methaemoglobin used in our experiments was not crystallized or purified other than by alumina treatment, and the possibility exists that enzymes of the erythrocyte play a part in the destruction of the compound.

The transient spectrum formed when hydrogen peroxide is added to haemoglobin in dithionite solution at pH 8·5 (Dalziel & O'Brien, 1952*a*) shows maxima at 417 and 545 mμ. of similar intensity to those of methaemoglobin-hydrogen peroxide, but in contrast to the latter there is also a distinct maximum at 582 mμ., of slightly greater intensity than that at 545 mμ. The reproducibility of this spectrum under a variety of conditions suggest that it characterizes a single compound, and not a mixture, and spectrokinetic measurements show that its rate of formation is greater than that of methaemoglobin-hydrogen peroxide with the same initial peroxide and pigment concentrations at pH 8·5. To obtain further evidence regarding the identity or otherwise of the two compounds, we made preliminary spectrokinetic experiments on the reaction of methaemoglobin-hydrogen peroxide with excess of dithionite at pH 8·5. In the overall reaction some protohaem is lost, partly as cholehaem and partly as unidentified products. The spectrokinetic curves show that the formation of haemoglobin is preceded by a reaction involving general loss of absorption, and the duration and extent of this initial reaction, the overall loss of protohaem and the yield of choleglobin increase with increase of the initial peroxide concentration. Increase of the initial dithionite concentration curtails the initial reaction and the protohaem destruction but does not significantly affect the yield of choleglobin. Apparently in this initial period reaction occurs between methaemoglobin-hydrogen peroxide or an intermediate formed from it, and the excess of free hydrogen peroxide in the presence of dithionite, possibly by a peroxidative mechanism as suggested by Keilin & Hartree (1935), in which some of the

pigment undergoes oxidative degradation to choleglobin and other products.

This initial reaction is followed, presumably after destruction of the free hydrogen peroxide, by the slow reduction of the remaining methaemoglobin-hydrogen peroxide to haemoglobin. The spectrokinetic curves for 430 mμ. were logarithmic, and showed that the rate of haemoglobin formation increases with the initial dithionite concentration to a limiting value for the half-reaction time of 50 sec. with 0·8–2·0% (w/v) dithionite. The spectrokinetic curves for 580 mμ. did not show any intial increase of extinction such as would suggest the intermediate formation of the transient spectrum observed in the reaction of haemoglobin, dithionite and peroxide. In this latter reaction, the rate of reversion of the transient compound to haemoglobin also increases with the initial dithionite concentration, to a limiting value for the half-reaction time of 25 sec. with 1·6–2·0% dithionite, and appears, therefore, to be significantly faster than the reduction of methaemoglobin-hydrogen peroxide. We hope to report experimental evidence regarding the nature of this transient compound shortly.

SUMMARY

1. The reaction between catalase-free human methaemoglobin and hydrogen peroxide has been studied spectrokinetically. With high concentrations of peroxide at pH's 6·0 and 8·5, methaemoglobin is completely converted into methaemoglobin-hydrogen peroxide, which then undergoes a relatively slow consecutive reaction. The absorption spectrum of the compound, which shows maxima at 418 mμ., $\epsilon_{mEq.} = 105$ and 545 mμ., $\epsilon_{mEq.} = 10{\cdot}5$, is not affected by pH in the region 6·0–8·5.

2. Reasonably reliable kinetic data for the formation of methaemoglobin-hydrogen peroxide have been deduced from the spectrokinetic data of experiments in which the hydrogen peroxide was present in several excessive concentrations. The rate increases with the hydrogen-ion concentration, the velocity constant at 17° being 18 M^{-1} $sec.^{-1}$ at pH 8·5 and 220 M^{-1} $sec.^{-1}$ at pH 6·0. Spectrokinetic data for the reaction at pH 6·0 with low peroxide concentrations are consistent with a reversible reaction for which $pK = 4{\cdot}4$ at 23°. The results are discussed in relation to recent work on the reaction of metmyoglobin with hydrogen peroxide.

3. Some observations on the reaction of methaemoglobin-hydrogen peroxide with sodium dithionite at pH 8·5 are described. The compound is mainly reduced to haemoglobin in a relatively slow reaction, but some protohaematin is degraded to cholehaem and other products in a rapid initial reaction with the excess of hydrogen peroxide.

4. Comparative spectral and kinetic data for methaemoglobin-hydrogen peroxide and the transient compound formed by the action of hydrogen peroxide on haemoglobin in the presence of dithionite are briefly discussed.

5. The absorption maxima and extinction coefficients of human methaemoglobin in acid and alkaline solution are recorded.

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Spectrophotometric Studies of the Reaction of Methaemoglobin with Hydrogen Peroxide

2. THE DEGRADATION OF METHAEMOGLOBIN BY HYDROGEN PEROXIDE

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Keilin & Hartree (1935) showed spectroscopically that methaemoglobin-hydrogen peroxide, formed by the addition of hydrogen peroxide to catalase-free solutions of horse methaemoglobin at pH 5·8–6·5, decomposed on standing with the liberation of acid methaemoglobin, the peroxide being destroyed without oxygen evolution, presumably in true oxidative degradation of that part of the methaemoglobin which was lost during the reaction. Later, more detailed observations of the formation and decomposition of the compound were reported (Keilin & Hartree, 1951). In presence of an excess of peroxide in the pH range 5·5–9·2, the formation of oxyhaemoglobin was detected, followed by decomposition of the hydrogen peroxide with oxygen evolution, and the eventual destruction of the pigment to a colourless solution. In solutions more acid than pH 5, with smaller peroxide concentration, the formation of oxyhaemoglobin was not observed, and the reaction yielded a red-green solution with a wide absorption band at 590 mμ.

According to Lemberg, Legge & Lockwood (1941) hydrogen peroxide reacts with methaemoglobin, oxyhaemoglobin and haemoglobin to form choleglobin, a green haem pigment first characterized in the products of the coupled oxidation of oxyhaemoglobin and ascorbic acid (Lemberg, Legge & Lockwood, 1938, 1939). These workers suggested a compound of haemoglobin and hydrogen peroxide to be the precursor of choleglobin. In the reaction of hydrogen peroxide with haemoglobin in the presence of sodium dithionite at pH 8·5, which also yields choleglobin, we have observed the conversion of haemoglobin into an unstable intermediate compound (Dalziel & O'Brien, 1951, 1952*a*). The similarity between the spectral absorption of this transient compound and that of methaemoglobin-hydrogen peroxide, led to the experiments on the formation and breakdown of the latter compound described in this and in the preceding paper.

In this paper, detailed spectrophotometric studies of the reaction of methaemoglobin with hydrogen peroxide are described. At pH 6, with the peroxide in moderate excess, a stable green pigment distinct from choleglobin is formed, and yields a carbon monoxide derivative in the presence of sodium dithionite. The formation of the green pigment is accompanied by the progressive transformation of the protohaematin into a new haem which will form stable haemochromogen derivatives. Complete conversion of protohaematin into the green haem was not achieved: with low peroxide concentrations, the reaction was apparently quantitative although incomplete, and with higher peroxide concentrations both the protohaematin and the green haematin were destroyed. This work has already been published in the form of an Abstract (Dalziel & O'Brien, 1952*b*).

EXPERIMENTAL

Spectrokinetic measurements

Measurements of spectral absorption were made with a Beckman quartz photoelectric spectrophotometer, model D.U., and the results are plotted as the extinction, $E = \log I_0/I$. The reactants were mixed in optical cells of 1 cm. or 0·5 cm. path length, and the time course of the extinction at two or three wavelengths was followed by making measurements in rapid succession from about 1 min. after mixing. This was repeated at other wavelengths with fresh reaction mixtures. The complete absorption spectrum of the mixture at any time after mixing could be built up from the plots of extinction against time for many wavelengths. In the later stages of the reaction, when the light absorption was changing but slowly, the whole spectrum was rapidly scanned back and forth, so that the means of the pairs of extinction measurements corresponded to the same time after mixing.

Reagents and derivatives

Hydrogen peroxide. A.R. '20 vol.' H_2O_2 was diluted as required with Clarke & Lubs's buffer solutions (cf. Vogel, 1939), 0·05 M phosphate:NaOH, pH 6·0, or 0·05 M borate:NaOH pH 8·5. The stock solution was repeatedly standardized against $KMnO_4$.

Methaemoglobin (metHb). This was prepared from haemolysates of washed human red cells by oxidation with $K_3Fe(CN)_6$, after removal of the catalase by adsorption on alumina; the concentration of metHb solutions was estimated by conversion into the cyanide (Drabkin & Austin, 1935), and is expressed as m-equiv./l. (mEq.), where 1 mEq. metHb = 1 mM haematin. Details of the preparation and analysis were given previously (Dalziel & O'Brien, 1954).

Denatured globin carboxyhaemochromogen derivatives. A very slight modification of the procedure described by Lemberg *et al.* (1941) was used. Coal gas was rapidly passed into 5 ml. metHb solution, or metHb and H_2O_2 mixture, in a test tube for 15 sec., and approx. 50 mg. $Na_2S_2O_4$ were then added; the tube was inverted twice, and whilst coal gas was again passed slowly into the mixture, 0·5 ml. 5N-NaOH was added down the wall of the tube. The mixture was quickly rolled round the wall of the tube, and finally coal gas was passed again for 1 min. The method gave fairly reproducible results, and tests of its validity in the presence of free H_2O_2 are described below. For brevity, such preparations will be referred to as CO-haemochromogens.

RESULTS

Spectrokinetic observations

The changes of light absorption accompanying the overall reaction of metHb and H_2O_2 at pH's 6·0 and 8·5 were recorded in optical cells. The reaction mixtures were initially about 0·06 mEq. with respect to metHb and various peroxide concentrations were used. The formation of methaemoglobin-hydrogen peroxide and the early stages of its subsequent reaction were described previously (Dalziel & O'Brien, 1954).

At pH 8·5 with initial peroxide/haematin ratios (R) of 4·5, 18 and 44, the spectrum of methaemoglobin-hydrogen peroxide first formed, with a maximum at 545 mμ. and a pronounced shoulder at 570–580 mμ., slowly decreased in intensity, and the absorption maximum at 545 mμ. gradually shifted back to 540 mμ. The final products with the lower peroxide concentrations showed depressed maxima at 408, 546 and 576 mμ., consistent with partial reversion to metHb, and on addition of dithionite gave a haemoglobin spectrum. The spectra of CO-haemochromogen derivatives of the products were those of CO-protohaemochromogen with a very small additional maximum at 625–630 mμ. The extents of protohaematin and metHb destruction during the reaction, calculated from the absorption spectra, were about the same, and increased with the initial peroxide concentration. Evidently the decomposition of the compound in presence of excess of peroxide under these conditions is accompanied by disintegration of part of the protohaematin, without the formation of appreciable quantities of stable intermediate pigments.

In weakly acidic conditions, with not too great an initial concentration of peroxide, at least one stable pigment other than metHb is formed, and the remainder of this paper is devoted to the reaction at pH 6·0. In Fig. 1, absorption curves are shown for three 0·059 mEq. metHb solutions containing various amounts of peroxide. After 2 min., the spectrum of each reaction mixture is qualitatively that of methaemoglobin-hydrogen peroxide (Keilin & Hartree, 1951), although with the lowest peroxide concentration, $R = 4·5$ (Fig. 1*a*), compound formation is incomplete. The subsequent course of the reaction depends upon the peroxide concentration. With $R = 91$, Fig. 1*c*, the specific absorption is gradually destroyed, and the spectrum of the compound persists throughout the degradation. With $R = 4·5$, the colour of the mixture slowly reverts from red to brown; the changes of spectral absorption (Fig. 1*a*) in the region 500–550 mμ. are broadly consistent with partial reversion to metHb, but the changes at longer wavelengths, and the spectrum of the stable products (85 min.), show that one or more other substances with significant absorption at 520–630 mμ. are also formed. With $R = 18$, Fig. 1*b*,

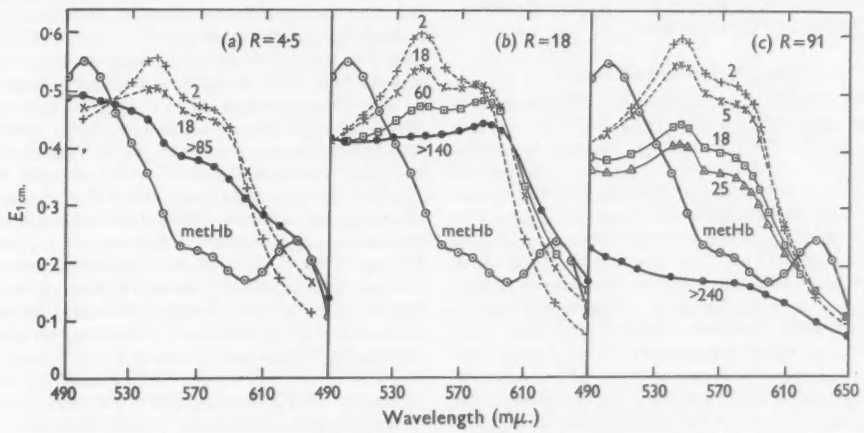

Fig. 1. Absorption spectra of reacting mixtures of metHb and H_2O_2 at intervals (min.) after mixing. MetHb: 0·059 mEq. H_2O_2: (*a*) 0·265 mM, (*b*) 1·06 mM, (*c*) 5·37 mM, pH 6·0.

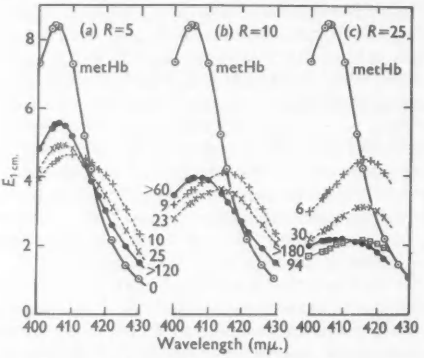

Fig. 2. Spectral changes with time (min.) in the Soret region during the later stages of the reaction of 0·05 mEq. metHb with H_2O_2: (*a*) 0·25 mM-H_2O_2, (*b*) 0·50 mM-H_2O_2, (*c*) 1·25 mM-H_2O_2. pH 6·0.

there are no clear indications of metHb formation, and whilst the maximum at 545 mμ. is destroyed, a broad peak at 585 mμ. emerges; the reaction yields a green-brown solution.

In Fig. 2, the corresponding changes in the Soret region are shown for 0·050 mEq. metHb solutions with $R = 5$, 10 and 25. The early stages of the reaction, the partial transformation of the metHb into the compound, $\lambda_{max.}$ 418 mμ., have been described previously (Dalziel & O'Brien, 1954) and are not shown. With the lowest peroxide concentration partial reversion to metHb is apparent. With the higher peroxide concentrations, destruction of methaemoglobin-hydrogen peroxide is accompanied by decreasing absorption from 400–430 mμ., followed by increasing absorption near 405 mμ., again consistent with some reversion to metHb in the final stages of the reaction.

Reaction of the protohaematin

After treatment with dithionite in the presence of carbon monoxide, the green products of the reaction with $R = 18$, described above, showed a typical carboxyhaemoglobin spectrum with an additional pronounced maximum at 613 mμ.; further treatment with alkali and carbon monoxide gave a CO-protohaemochromogen spectrum with an additional maximum at 617 mμ. The absorption intensities in both cases indicated loss of protohaematin during the reaction with peroxide. Similar derivatives were obtained from the reaction products with $R = 4·5$, but the new maxima were less intense and the protohaematin losses smaller. These observations suggested that part of the protohaematin had been converted into a derivative of the choleglobin type.

The progress of the reaction of the prosthetic group was followed by withdrawing samples from reacting mixtures of metHb and peroxide at intervals, converting into CO-haemochromogens by treatment with coal gas, dithionite and alkali, and recording the absorption spectra. This technique was used by Lemberg *et al.* (1941) in quantitative studies of the conversion of protohaem into cholehaem in mixtures of oxyhaemoglobin and ascorbic acid; the formation of choleglobin during the haemochromogen preparation, by the rapid reaction of haemoglobin with hydrogen peroxide formed by autoxidation of the dithionite, was prevented by saturating the test samples with coal gas before adding the dithionite. The validity of the method in its present application to mixtures of metHb and peroxide was confirmed by the following observations: (1) Carboxyhaemoglobin solutions to which peroxide was added ($R = 18$ and 91) yielded quantitative CO-haemochromogen spectra. (2) The absorption spectra of CO-haemochromogens prepared from metHb solutions immediately after the addition of peroxide ($R = 4·5$) by treatment with dithionite, alkali and coal gas, in that order, showed a significant loss of protohaematin and a pronounced CO-cholehaemochromogen maximum at 630 mμ. When the metHb and peroxide solutions were saturated with coal gas before mixing, similar treatment yielded a quantitative CO-haemochromogen spectrum. (3) Treatment of a reaction mixture of metHb and peroxide ($R = 18$) with coal gas immediately after mixing had no effect on the slow reaction involving the protohaematin described below, as judged by CO-haemochromogen spectra of samples withdrawn at intervals up to 20 min. after mixing. Accordingly, in order to study the reaction during the first minute or two after mixing, the reactants were separately saturated with coal gas before mixing, and the reaction was quenched at the desired time by the addition of dithionite. This last observation suggests that oxyhaemoglobin is not an intermediate in the formation of the green pigment.

The absorption spectra of CO-haemochromogen derivatives prepared from the three reaction mixtures whose spectral absorptions were given in Fig. 1, are shown in Fig. 3. With $R = 45$ and $R = 18$, Fig. 3*a*, *b*, the decline of the CO-protohaemochromogen maxima at 539 and 568 mμ. is accompanied by the progressive elevation of a peak at 617 mμ. Since there is an isosbestic point at 585 mμ. (and at 510 mμ.) in both sets of curves, and the increases of $E617$ (i.e. E at 617 mμ.) for given decreases of $E568$ are about equal, the partial conversion of protohaematin into a new, stable haematin apparently occurs without much loss by other reactions. In the mixture with $R = 91$, however, the haem structure is evidently rapidly destroyed: the spectra, Fig. 3*c*, do not show an isosbestic point, and a limited increase of absorption around

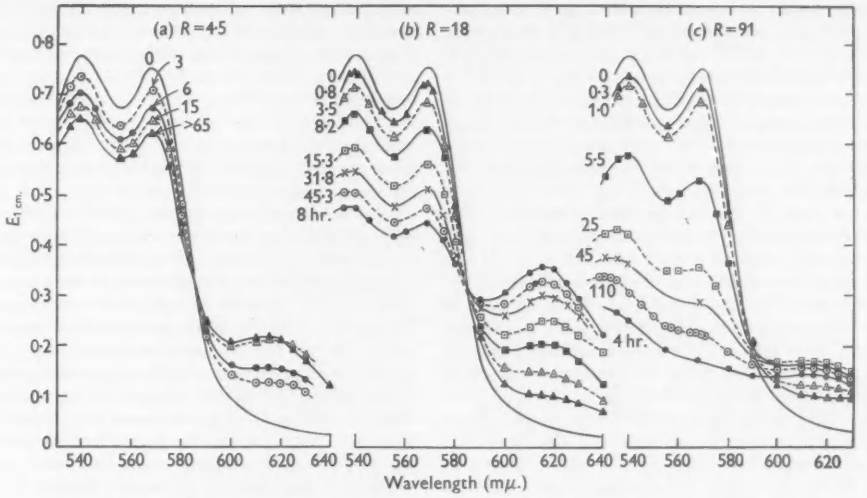

Fig. 3. Absorption spectra of CO-haemochromogen derivatives prepared at intervals (min.) during the reaction of 0·059 mEq. metHb with H_2O_2: (*a*) 0·265 mM-H_2O_2, (*b*) 1·06 mM-H_2O_2, (*c*) 5·37 mM-H_2O_2. pH 6·0.

617 mμ. in the first few minutes is partly destroyed subsequently.

The time course of the protohaematin reaction (Fig. 3) throws some light on the concurrent, more complex changes in the absorption spectra of the reaction mixtures (Fig. 1). With $R=91$, it is evident that during the degradation the haem exists mainly as protohaematin in the form of methaemoglobin-hydrogen peroxide. With $R=18$, the reaction involving the protohaematin lasts about 1 hr., and is associated with the emergence in the pigment spectrum (Fig. 1*b*) of the maximum at 585 mμ. and also with decreasing absorption in the Soret region (Fig. 2*b*, *c*). The more uniform subsequent decline of the pigment spectrum in the region 500–600 mμ., ultimately quite extensive, is not accompanied by significant changes in the CO-haemochromogen spectrum (Fig. 3*b*), and is doubtless in part due to reversion of the remaining methaemoglobin-hydrogen peroxide to metHb; the terminal changes in the Soret spectrum (Fig. 2*b*, *c*) support this interpretation. Similarly, with $R=4·5$ the changes in the pigment spectrum are satisfactorily accounted for by the reaction involving the protohaematin, occurring mainly in the first 15 min. (Fig. 3*a*), accompanied and followed by reversion of the compound to metHb.

The effect of hydrogen peroxide concentration on the nature of the products

The complexity of the reaction at pH 6·0 is apparent when the absorption spectra of the final products of the reaction of 0·059 mEq. metHb with

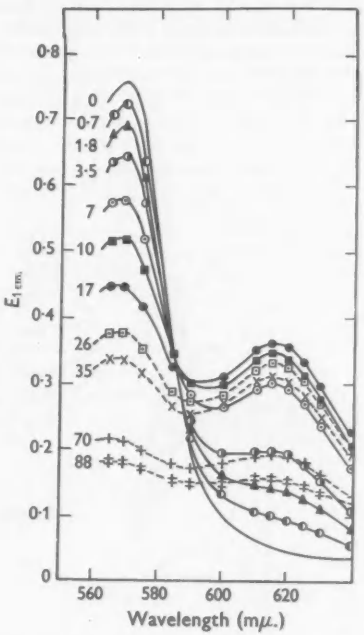

Fig. 4. Absorption spectra of CO-haemochromogens prepared from the products of the reaction of 0·059 mEq. metHb with various amounts of H_2O_2, expressed as R=initial peroxide/haematin molar ratio. Full lines, R=0–17; broken lines, $R>17$.

various amounts of peroxide and of the corresponding CO-haemochromogen spectra are examined. The group of CO-haemochromogen curves (Fig. 4) for peroxide concentrations from $R=0{\cdot}7$ to $R=10$ shows an isosbestic point at 584 mμ. and is consistent with a series of mixtures of constant total concentration of protohaemochromogen, $\lambda_{max.}=568$ mμ., and the haemochromogen responsible for the peak at 617 mμ. With R values greater than 17, the CO-haemochromogen spectra have progressively smaller specific absorption, and the residual maxima at 539 and 568 mμ. and the new maximum at 617 mμ. are affected to about the same extent. The Soret spectrum of the derivatives from the products of the reaction with $R=17$ and $R=88$ were also recorded, and showed peaks at 419 mμ. consistent with decreasing amounts of residual CO-protohaemochromogen.

The absorption spectra of the products themselves, some of which are shown in Fig. 5, form a more complicated series, but a discontinuity at $R=10$–17 can again be distinguished. As R is increased from 0·7 to 10 the metHb maximum at 500 mμ. is progressively destroyed and a new peak emerges at about 580 mμ. There are no isosbestic points, however, and since the haemochromogen spectra suggest that there is no significant total haem destruction in these mixtures, the metHb is evidently transformed into more than one product in this range of peroxide concentration; a small peak at 530 mμ. in the products with $R=7$ is noteworthy in this connexion. In the Soret region the intensity decreases rapidly with increase of peroxide concentration and the maximum shifts slightly towards the red. The products with $R=17$ show maxima at 585 and 408 mμ., the latter having about two-fifths the intensity of the original metHb Soret band, which bands persist with decreasing intensity in the products with higher peroxide concentrations as the specific absorption is destroyed.

It is concluded that in the overall reaction the protohaematin of metHb is partially transformed by peroxide into a new haem in the form of a green pigment which has an absorption maximum at 585 mμ. and much smaller absorption than metHb in the Soret region. With peroxide concentrations up to $R=10$ the reaction is apparently almost quantitative, but with higher peroxide concentrations haem destruction occurs to an increasing extent. The yield of green haem is greatest with $R=10$–17, but even with higher initial peroxide concentrations unchanged protohaematin apparently persists, and might account for the residual Soret band observed in the spectra of the products and derivatives.

The effect of repeated reaction with hydrogen peroxide

The complete conversion of protohaematin into the green haem was attempted by successive treatment of 0·059 mEq. metHb with three portions of 7 equiv. each of peroxide, added as a concentrated solution to avoid appreciable dilution of the pig-

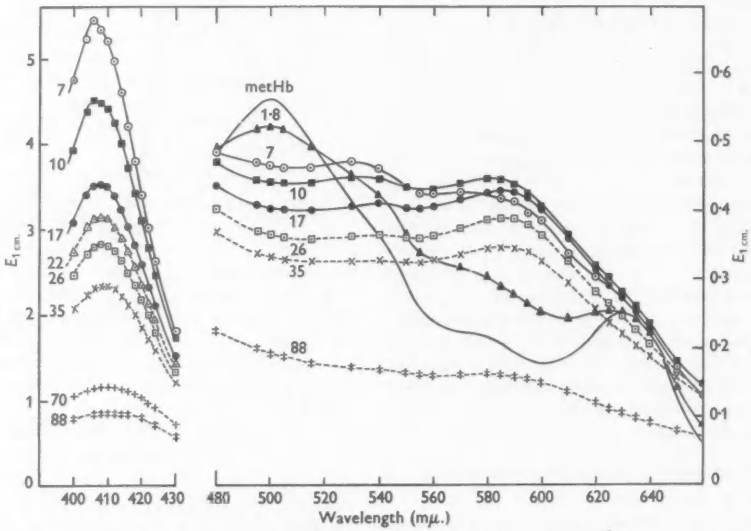

Fig. 5. Absorption spectra of the products of the reaction of 0·059 mEq. metHb with various amounts of H_2O_2, expressed as R = initial peroxide/haematin molar ratio. Full lines, $R=0$–17; broken lines $R>17$.

ment solution. After each addition of peroxide the reaction was followed spectrokinetically at 500–630 mμ. until the spectrum became stable. A sample was then withdrawn before the next addition of peroxide, and converted into CO-haemochromogen derivatives.

The second 7 equiv. of peroxide caused immediate changes in the spectrum of the pigment consistent with the formation of some methaemoglobin-hydrogen peroxide, the increase of E 545 and E 576 corresponding to the conversion of about 25% of the original metHb into this compound. When stable, the absorption spectrum had a distinctly sharper and more intense maximum at 585 mμ. than that obtained by a single reaction with 15 equiv. of H_2O_2. The CO-haemochromogen spectrum showed a corresponding enhancement of the 617 mμ. maximum at the expense of the CO-protohaemochromogen maxima, but there was also a small decrease of intensity at the isosbestic point, 584 mμ., and the ratio of the decrease of E 568 to the increase of E 617 was significantly greater than in the reaction with the first 7 equiv., showing that some protohaematin had been lost in the second stage as products other than the green haem. Residual maxima still remained, however, at 539 and 568 mμ. The third 7 equiv. of peroxide produced only small immediate changes in the pigment spectrum, which corresponded to the conversion of about 2% residual metHb into methaemoglobin-hydrogen peroxide and the reaction resulted ultimately in small, uniform decreases of intensity in both pigment and CO-haemochromogen spectra, without change of shape, which indicated some destruction of total haem, including green haem.

In a similar experiment, three successive reactions with 5 equiv. of peroxide were carried out. In this case, the third reaction also produced a significant amount of methaemoglobin-hydrogen peroxide and ultimately an increase in the yield of green pigment. The final pigment and CO-haemochromogen spectra agreed closely with those obtained after two reactions with 7 equiv. of peroxide.

Properties of the products

The reaction of 0·059 mEq. metHb with two successive portions of 7 equiv. of peroxide seemed to give the maximum yield of green pigment, and apparently there was little unchanged metHb. The green-brown solution was allowed to stand for several hours at room temperature and then overnight in the cold store. The absorption spectrum in the visible region is shown in Fig. 6. The Soret band at 408 mμ. had about one-third the intensity of the original metHb peak at 405 mμ. The effect of various reagents on the spectrum were studied and the absorption curves of some of the derivatives are given in Figs. 6 and 7.

Carbon monoxide produced no change in the absorption spectrum other than a small uniform decrease of intensity.

Cyanide and fluoride. The addition of 0·1 ml. 0·3% potassium cyanide to 5 ml. pigment solution produced only a slight change in the spectrum; the increase of E 545 indicated a residual metHb content of 8% of the original. The products of the reaction with a single 7 equiv. of peroxide when similarly

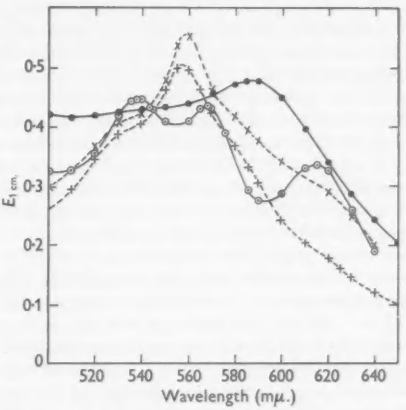

Fig. 6. Effect of $Na_2S_2O_4$ and $CO + Na_2S_2O_4$ on the absorption spectrum of the products of the reaction of 0·059 mEq. metHb successively with two portions of 7 equiv. each of H_2O_2: ●—●, reaction products; × - - - ×, 2 min., and + - - - +, 2 hr., after addition of $Na_2S_2O_4$; ⊙—⊙, $CO + Na_2S_2O_4$.

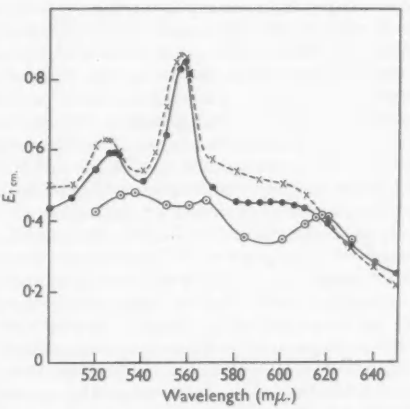

Fig. 7. Haemochromogen derivatives of the products of the reaction of 0·059 mEq. metHb successively with two portions of 7 equiv. each of H_2O_2: ●—●, denatured globin haemochromogen; ⊙—⊙, denatured globin CO-haemochromogen; × - - - ×, pyridine hemochromogen.

treated yielded a spectrum with a broad maximum at 540–550 mμ., consistent with the presence of 33% residual metHb, and the crossing-points with the pigment spectrum were in good agreement with those of metHb and its cyanide derivative, confirming that the green pigment does not react with cyanide. With fluoride similar results were obtained; the extinction changes indicated the presence of 32 and 4% residual metHb in the products after the first and second reaction, respectively.

Haemochromogens. Pyridine haemochromogens were prepared by diluting 2·5 ml. pigment solution with 1·4 ml. phosphate buffer, pH 6, and adding 0·1 ml. 5N-NaOH, 1 ml. pyridine and 50 mg. dithionite. Denatured globin haemochromogens were prepared by diluting 2·5 ml. pigment solution with 2 ml. buffer and adding 50 mg. dithionite and 0·5 ml. 5N-NaOH. Denatured globin carboxyhaemochromogens were prepared as described previously. The absorption spectra were recorded in 1 cm. cells sealed with liquid paraffin and a cover glass, and were stable for at least an hour. The results, corrected for dilution, are given in Fig. 7. Corresponding to the maximum at 617 mμ. in the CO-haemochromogen spectrum, the haemochromogen spectra each have a shoulder at about 610 mμ.: the direct conversion of the denatured globin haemochromogen spectrum into that of the carbon monoxide derivative was confirmed. All three spectra show distinct residual maxima in the green corresponding to those of protohaematin derivatives.

Reduction in presence of carbon monoxide. The absorption curve obtained on addition of 50 mg. dithionite to 5 ml. pigment solution after saturation with coal gas is shown in Fig. 6. The bands in the green correspond with those of carboxyhaemoglobin, and that in the red is centred at 613 mμ. When dithionite was added to the pigment in absence of carbon monoxide, a slow reaction occurred which is described below. Carbon monoxide and CO-haemochromogen derivatives prepared 30 min. or more after the addition of dithionite showed no peaks in the red, but the two-banded spectrum in the green remained unchanged.

Reaction with dithionite. When a little dithionite was added to the green-brown pigment solution, the brown tinge was destroyed, and spectroscopic observation showed that the broad, diffuse band at 592 mμ. was replaced by a sharper band at 560 mμ. After exposure to air with gentle shaking, the colour of the solution changed from green to pale red-brown, and the band at 560 mμ. was replaced by two bands at 544 and 578 mμ. This reaction was reversed by addition of more dithionite, and ferricyanide also destroyed the two-banded spectrum.

These reactions were studied more fully with the spectrophotometer. Approximately 12 mg. dithionite were weighed into a dry 1 cm. cell, 3 ml. pigment solution pipetted in, and a layer of paraffin added. After being mixed with a fine glass stirrer, the cell was completely filled with paraffin and sealed with a cover slip. The extinction was recorded at three or four wavelengths in rapid succession and later at more wavelengths as the reaction slowed down. Similar measurements were made at other wavelengths with fresh preparations. The integrated spectral absorption of the mixture after 2 min. (Fig. 6), shows the immediate formation of a maximum at 558–560 mμ. and a marked inflexion at 530 mμ., which persists for longer than 2 hr. whilst the intensity of absorption gradually decreases, especially in the region 560–640 mμ. The carbon monoxide and CO-haemochromogen derivatives no longer show a band in the red. Evidently the green haem is destroyed in this reaction.

Reduction of the pigment solution without destruction of the green haem was effected by the addition of the minimum quantity of dithionite and oxidation of the excess by aeration. The pH of the pigment solution was first raised to about 7, and traces of dithionite were added until spectroscopic observation showed that the band at 592 mμ. had been replaced by one at 560 mμ. The absorption spectrum of a sample was briefly checked in the spectrophotometer, and the remainder was exposed to air with gentle agitation. The spectrum of the red-brown solution obtained, with peaks at 539 and 576 mμ., was quite stable. On treatment with coal gas the peak at 576 mμ. shifted to 569 mμ. and a distinct inflexion appeared at 610–615 mμ. The addition of dithionite restored the peak at 558 mμ. and the inflexion at 530 mμ., and the absorption spectra of denatured globin haemochromogen and CO-haemochromogen derivatives were similar to those obtained directly from the products of the reaction of metHb with peroxide, showing bands in the red. It appears that the green haem has not been completely destroyed, but now reacts with carbon monoxide.

DISCUSSION

In the overall reaction of human methaemoglobin with hydrogen peroxide at pH's 6·0 and 8·5, protohaematin was lost to an extent which increased with the initial hydrogen peroxide concentration. With a great excess of peroxide, the reaction ultimately yielded colourless products, but with smaller initial peroxide concentrations part of the methaemoglobin was recovered. Spectrokinetic studies suggested that the prosthetic group of the methaemoglobin-hydrogen peroxide first formed, which yields protohaem derivatives in the presence of sodium dithionite, underwent a slow reaction, presumably with the excess of hydrogen peroxide,

and that if the initial peroxide concentration were not too great, the remaining methaemoglobin-hydrogen peroxide reverted to methaemoglobin. These findings are in accord with those of Keilin & Hartree (1935, 1951) for horse methaemoglobin. At pH 8·5, neither the reaction mixtures nor their CO-haemochromogen derivatives gave spectrophotometric evidence for the occurrence of significant amounts of any haem compounds other than methaemoglobin-hydrogen peroxide and methaemoglobin. The reaction at pH 6, however, yielded a stable green pigment with a broad absorption maximum at about 585 mμ., and the spectra of derivatives of the products showed that part of the protohaematin had been converted into a new haem distinct from cholehaem.

Comparison of the absorption spectra of reacting mixtures with those of CO-haemochromogen derivatives prepared at intervals during the reaction (Figs. 1–3), and of the spectra of the products obtained with a range of initial peroxide concentrations with those of CO-haemochromogen derivatives (Figs. 4, 5) established the connexion between the green pigment and the new haem, and threw some light on the course of the reaction. With an initial methaemoglobin concentration of 0·059 m-equiv./l., the yield of green pigment increased with the initial peroxide concentration up to a peroxide/haematin ratio of about 15. Within this range the presence of an isosbestic point in the haemochromogen spectra suggested that protohaematin was progressively transformed into the green haem without appreciable loss by destruction of the haem nucleus or by other reactions, but a source of protohaemochromogen apparently remained in the products. The latter could account for the residual Soret bands, and it was evident from the rapid fall of absorption during the reaction that the green pigment and its CO-haemochromogen have relatively small absorption in the Soret region. There was evidence, particularly in the time course of the spectrum in the Soret region (Fig. 2), that when the excess of hydrogen peroxide had been used up in green pigment formation, some methaemoglobin was regenerated. There was evidence also, in the spectrum of the products with low peroxide concentrations, of the formation of at least one pigment other than methaemoglobin and green pigment which also yields the CO-haemochromogen of either protohaem or green haem. With peroxide/haematin molar ratios greater than 15, disintegration of the haem structure occurred to an increasing extent, and the yield of green pigment decreased. During the degradation to colourless products which occurs with great excess of peroxide, the intact haem was present very largely as the prosthetic group of methaemoglobin-hydrogen peroxide (Figs. 1 and 3, $R=91$); the intermediate formation of oxyhaemoglobin, observed by Keilin & Hartree (1951) with horse methaemoglobin in much greater concentration, was not detected.

Successive reaction of methaemoglobin with hydrogen peroxide gave a slightly greater maximum yield of green pigment than could be obtained in a single reaction, but again, haemochromogen spectra showed distinct protohaemochromogen bands which could not be destroyed by further addition of peroxide to the products without concomitant destruction of green haem. From the effects of cyanide, fluoride and hydrogen peroxide on the spectrum of the products, it was estimated that about 5% of the original methaemoglobin remained. In the absorption spectra of pyridine and denatured globin haemochromogens, the pronounced residual bands in the green attributed to protohaem were apparently undistorted, and on the reasonable assumption that the absorption spectra of the green haem derivatives were flat in this region, it was estimated from the height of the maxima above the central minimum that about 25% of the initial protohaem was recovered, an amount significantly greater than the estimated methaemoglobin recovery.

Carbon monoxide alone had no specific effect on the spectrum of the products, but sodium dithionite in the presence of carbon monoxide yielded a stable spectrum comprising typical undistorted carboxyhaemoglobin maxima at 539 and 568 mμ., and a maximum at 613 mμ. due to the green pigment derivative; from the height of the bands in the green above the saddle it was estimated that about 25% of the initial methaemoglobin was thus recovered as carboxyhaemoglobin. In the absence of carbon monoxide the green pigment was slowly destroyed by dithionite, as shown by the disappearance of the bands in the red in the haemochromogen and carbon monoxide derivatives, but the peaks in the green were unaffected. After reaction with dithionite, the spectrum of the products resembled that of haemoglobin, but in addition to a maximum at 560 mμ., there was a pronounced inflexion at about 530 mμ. These observations suggest that oxyhaemoglobin may be the source of the residual protohaem in the products, but further evidence is required on this point.

The prosthetic group of the green pigment yields stable pyridine and denatured globin haemochromogens with bands in the red, which appear in the absorption spectra of derivatives of the reaction products as distinct shoulders at 600–610 mμ. (Fig. 7); the denatured globin haemochromogen forms a carbon monoxide derivative with a maximum at 617 mμ. The green pigment itself is more difficult to characterize because of the presence of other pigments in the products and because it undergoes a reaction with dithionite in which the

green haem is probably destroyed; it has an absorption maximum in the neighbourhood of 585 mμ., and relatively small absorption in the Soret region. Its most definite characteristic is the formation of a stable derivative on treatment with dithionite in the presence of carbon monoxide, with an absorption maximum at 613 mμ. Carbon monoxide alone has little effect on the spectrum of the green pigment. It is probable, therefore, that the pigment is a ferric compound, although it did not appear to react with cyanide or fluoride. There were indications that a stable form which would react with carbon monoxide could be obtained by treatment with a trace of dithionite.

The mode of preparation and the spectral characteristics of the green pigment and its derivatives suggest that it is yet another green haemoglobin of the choleglobin type. We hope to investigate its relationship to other members of the group and to verdohaem and biliverdin in future work. Comparison with the results of other workers also reveals some anomalies which require resolution. Lemberg *et al.* (1941) reported that choleglobin was formed in the reaction of methaemoglobin with hydrogen peroxide, but experimental details were not given; it was suggested that methaemoglobin was reduced to haemoglobin by the peroxide, which was then converted into a haemoglobin-hydrogen peroxide compound the precursor of choleglobin (Lemberg *et al.* 1941; cf. Dalziel & O'Brien, 1952*a*). Since we have found that carbon monoxide does not inhibit the formation of 'green haem' from methaemoglobin, the view that only ferrous compounds are oxidized by hydrogen peroxide to 'bile pigment haem' derivatives (Lemberg & Legge, 1943) may require revision. Keilin & Hartree (1951) found a green pigment very similar in spectral characteristics to that which we have described, in the products of the reactions of recrystallized horse methaemoglobin and metmyoglobin and hydrogen peroxide at pH 4·5, with similar reactant concentrations to ours. At pH values greater than 5·0, however, with greater pigment concentration, green pigment formation was not reported; the formation of oxyhaemoglobin and oxymyoglobin was observed to precede the destruction of the haem nucleus. George & Irvine (1952), on the other hand, found that the formation of metmyoglobin-hydrogen peroxide at pH < 7·0 was accompanied by side reactions which appeared to yield a product similar to that which we have described, and the spectra of carboxyhaemochromogen derivatives showed a pronounced peak at 615 mμ. in addition to protohaemochromogen peaks. They concluded, however, that the side products were formed rapidly during the initial compound formation, and not by a slow, consecutive reaction of the compound, such as our experiments with methaemoglobin indicate; this difference may be due to the much greater peroxide/haematin molar ratios used in our experiments (George & Irvine, 1953).

The mechanism by which green pigment is formed is of interest in relation to peroxidatic activity. It is not clear why the conversion of protohaem into green haem, which appears to use hydrogen peroxide quite efficiently and is apparently the only reaction involving the prosthetic group at low peroxide concentrations, does not go to completion, and why, beyond a certain limit, further oxidation of protohaem is accompanied by destruction of the green haem. If the residual protohaem is indeed in the form of oxyhaemoglobin, it might be expected to yield methaemoglobin and subsequently more green pigment on addition of more hydrogen peroxide. There is, of course, the possibility of the formation of molecules containing both green haem and protohaem, and an attractive hypothesis is that smooth conversion of protohaem into green haem, presumably by oxidative attack at a methine linkage, is a form of intramolecular peroxidative reaction requiring at least one intact protohaem per molecule. The mechanism of the reaction and the nature of the residual protohaem deserve more detailed study.

SUMMARY

1. The reactions of human methaemoglobin and hydrogen peroxide at pH's 6·0 and 8·5, which yield initially methaemoglobin-hydrogen peroxide, have been studied throughout their course by spectrokinetic observations and by conversion into haemochromogen derivatives. With low peroxide concentrations, the compound reverted, in part, to methaemoglobin, but with high peroxide concentrations the haem structure was ultimately destroyed.

2. At pH 8·5, no evidence was obtained for the occurrence, in significant amount, of haem compounds other than methaemoglobin and methaemoglobin-hydrogen peroxide. At pH 6·0, a green pigment was formed, which, in the presence of sodium dithionite, yielded stable carbon monoxide and haemochromogen derivatives showing bands in the red. The pigment itself was not stable towards sodium dithionite.

3. The green pigment was formed by partial oxidative degradation of the prosthetic group of methaemoglobin-hydrogen peroxide, which yields protohaem derivation in the presence of sodium dithionite. The green pigment has not been obtained free from protohaem compounds; the yield increased with the initial peroxide concentration up to a certain limit, but beyond this point, even on successive reaction of the products with small quantities of peroxide, simultaneous destruction of the residual protohaem and the green haem occurred. The residual protohaem was not entirely

in the form of methaemoglobin, but was apparently accounted for as carboxyhaemoglobin after the addition of carbon monoxide and dithionite.

4. The green pigment is distinct from cholegloblin, and its formation is not prevented by carbon monoxide, showing that oxyhaemoglobin is not an intermediate in the reaction.

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Bile Pigment Formation *in vitro* from Haematin and Haem Derivatives

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In a previous study it was observed that haematin in aqueous medium can undergo coupled oxidation *in vitro* with ascorbic acid to yield a product which, on treatment with 66% acetic acid, was decomposed to bile pigments (Kench, Gardikas & Wilkinson, 1950). The formation of bile pigment *in vitro*, therefore, contrary to the views of Lemberg, Cortis-Jones & Norrie (1938), was not dependent on the iron of haem being in the ferrous state and coupled with pyridine or protein.

Other conclusions reached in the previous *in vitro* investigations were: (1) Ferrihaemoproteins, such as methaemoglobin and methaemalbumin, were as suitable sources of bile pigment as haemoglobin. There was no evidence that reduction to the corresponding ferrous compounds occurred during the reaction. (2) No bile pigments could be detected in experiments with free protoporphyrin, nor was any porphyrin found in the breakdown products of any haem derivative. (3) Coupling of haematin with protein improved the yield of bile pigment, but the effect was non-specific in that oxyhaemoglobin and methaemalbumin, with different proteins and types of linkage with the prosthetic group, were equally good sources. More recently, cytochrome *c* has proved completely resistant on incubation with ascorbic acid (Gardikas & Kench, 1952), horse-liver catalase and horse-radish peroxidase were good sources of bile pigment (Kench, 1952) and metmyoglobin provided the highest yield obtained from any haem derivative (Kench & Gardikas, 1952).

It is possible that such *in vitro* studies only remotely resemble bile pigment formation *in vivo* where enzymes are able to produce such profound effects. Even London's (1950) demonstration of the convertibility *in vivo* of injected haematin into bile pigments, however, still leaves open the questions whether methaemalbumin was formed as an intermediate, and to what extent this mechanism actually operates in the catabolism of haemoglobin.

To obtain more information on chemical factors which influence the degradation of natural haem derivatives, further experiments have been made on the *in vitro* conversion of haematin and haemoproteins into bile pigments, in which special attention has been paid to the spectroscopic detection of bile pigment intermediates. Evidence of such compounds was secured for myoglobin and haematin, and, tentatively, for horse-radish peroxidase. The final reaction mixture from haematin appeared to contain both verdohaematin and cholehaematin, possessed respectively of open and closed porphyrin structure (Lemberg & Purdom, 1949). The latter observations supported the conclusion that degradation of free haematin followed a course similar to that of haem combined in oxyhaemoglobin, in which the comparable intermediates were verdohaemoglobin and choleglobin.

EXPERIMENTAL AND RESULTS

Experiments with haematin. Haematin was prepared by the method of Nencki & Zaleski (1900) with purification as suggested by Duesberg (1934). The following experiments were made with aqueous haematin solutions in an attempt to detect spectro-

scopically intermediates in the catabolism of haematin to bile pigment.

Haemin (14 mg.) was dissolved in 10 ml. 5% (w/v) Na_2CO_3 and the pH brought to 8·0 by addition of 50 ml. 0·1 N HCl. This aqueous solution was incubated at 37° with ascorbic acid and continuously oxygenated by a vigorous stream of O_2. The effect on bile pigment formation of varying the incubation time and ascorbic acid concentration were studied in a series of experiments (Fig. 1).

Opening of the haem molecule, indicated by the diminution of optical density in the Soret region, was accompanied by increased spectral absorption in the red, not, however, proportional to the yield of bile pigment. The absorption band of ferrohaem at 595 mμ. was never observed. The solution was unbuffered, and it was observed that in the experiment in which 1000 mg. ascorbic acid were added continuously during 2 hr., 60 μg. free biliverdin (16% total yield), extractable with chloroform alone, were present. The final pH of the solution, measured by glass electrode, was 4·54. Experiments in which 100 mg. ascorbic acid only were added gave final pH 7·02, and no free bile pigment could be detected.

That the increased red absorption was not due to free biliverdin was clear from the experiment recorded in Fig. 3, in which 14 mg. haematin were incubated for 2 hr. following addition of 100 mg. ascorbic acid. The reaction products which had pH 5·5 gave a yield of 9·5 μg. biliverdin/ml. An aqueous solution of pure biliverdin of this concentration and pH was prepared, and its optical density in the red region measured. It was evident that the increased red absorption of the haematin reaction products could not be due to liberated biliverdin, even if all were free.

When the haematin : ascorbic acid products were treated with redistilled pyridine, two maxima rapidly appeared in the absorption curve, at 650 and 560 mμ. (Fig. 2). The latter is, undoubtedly, that of pyridine (ferro) haemochromogen, formed from residual ferrihaem in the presence of ascorbic acid. The peak at 650 mμ. corresponds with the maximum at 655 mμ. observed by Lemberg *et al.* (1938) for verdohaemochromogen, and supports the conclusion that one product in the coupled oxidation of free haematin and ascorbic acid must be similar to verdohaematin, which has been prepared in crystalline form by removal of pyridine from verdohaemochromogen (Lemberg & Legge, 1949), and shown to contain an opened porphyrin structure (Lemberg & Purdom, 1949). Verdohaematin dissolves in dilute

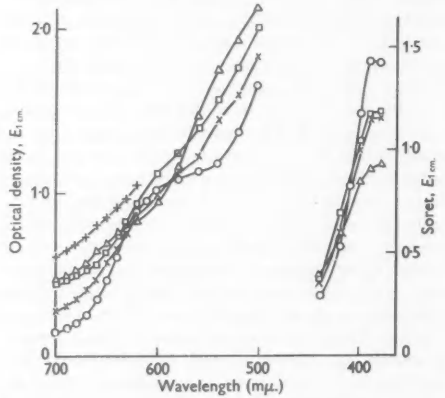

Fig. 1. Changes in the absorption spectrum of haematin on incubation with ascorbic acid. Haemin (14 mg.) was dissolved in 10 ml. 5% (w/v) Na_2CO_3, and the pH brought to 8·0 by addition of 50 ml. of 0·1 N-HCl: incubated at 37° with continuous rapid stream of O_2: ascorbic acid and incubation time varied as follows: O—O, haemin alone, 120 min., 0 μg. bile pigment. ×—×, +100 mg. ascorbic acid, 60 min., 237 μg. bile pigment. □—□, +100 mg. ascorbic acid, 120 min., 277 μg. bile pigment. △—△, +1000 mg. ascorbic acid, 120 min., 99 μg. bile pigment. +—+, +1000 mg. ascorbic acid added dropwise continuously, 120 min., 385 μg. bile pigment. The reaction mixture was examined undiluted for visual spectrum but diluted ×10 with water for Soret region.

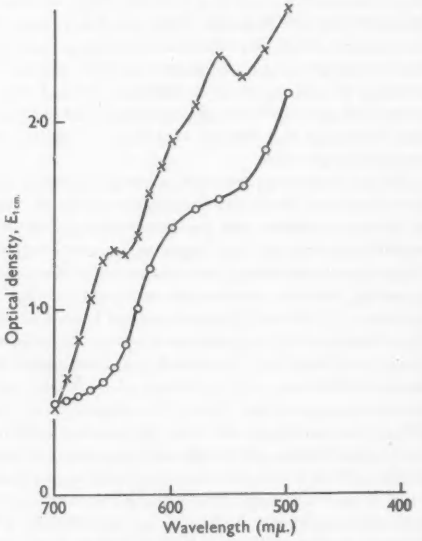

Fig. 2. Changes in the absorption spectrum of haematin: ascorbic acid reaction products following addition of pyridine. O—O, absorption spectrum of aqueous haematin (14 mg./60 ml. soln. pH 8·0) incubated 120 min. with 100 mg. ascorbic acid at 37° with passage of O_2. ×—×, 2 ml. final reaction mixture + 1 ml. redistilled pyridine. (*E* corrected for dilution with pyridine.)

sodium carbonate or phosphate buffer giving a dark-olive solution which absorbs light in the red, but shows no distinct absorption bands (cf. Fig. 1). Protein-free green haematins of the closed-ring 'chole' type have also been prepared (Lemberg & Purdom, 1949), but since choleglobin itself has not been prepared pure, no exact spectroscopic data are available for its prosthetic group, cholehaematin. Pyridine cholehaemochromogen has a maximum absorption at 619 mμ. (Lemberg, Legge & Lockwood, 1941*a*).

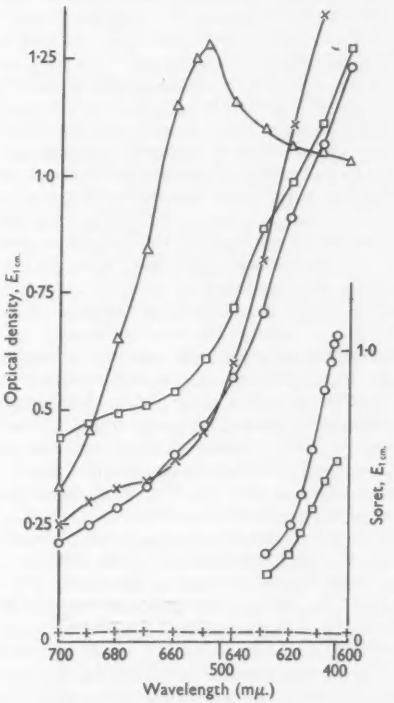

Fig. 3. Changes in the absorption spectrum of haematin: ascorbic acid reaction products following addition of pyridine. Experiments on the formation of a compound reacting with pyridine to give absorption at 650 mμ. with controls to check *de novo* formation of verdohaemochromogen, after addition of pyridine, by the action of residual ascorbic acid; latter determined by iodometric titration. ○—○, 14 mg. haematin incubated alone in O_2 for 120 min. then 0·5 mg. ascorbic acid added and absorption measured immediately. ×—×, 14 mg. haematin incubated alone in O_2 for 120 min. then 1 mg. ascorbic acid added per ml. and finally 1 ml. pyridine added to 2 ml. solution. Absorption curve measured immediately. □—□, 14 mg. haematin incubated with 100 mg. ascorbic acid at 37° for 120 min. with passage of O_2. △—△, 14 mg. haematin incubated for 120 min. at 37°, with O_2, and 600 mg. ascorbic acid added in first 60 min. Final solution treated with pyridine, and examined spectroscopically immediately. +—+, absorption of aqueous biliverdin 9·5 μg./ml. pH 5·5. Solutions were undiluted for visual spectrum, but diluted ×10 for the Soret region.

Control experiments (Fig. 3) have shown that the small amount of ascorbic acid remaining at the end of the experiment was not responsible for the production of verdohaemochromogen *de novo* from unchanged ferrihaem dissolved in pyridine. More vigorous treatment of haematin through addition of larger quantities of ascorbic acid during incubation, caused the absorption maximum with pyridine at 650 mμ. to become more prominent.

Treatment of the mixture, following addition of pyridine, with 66% acetic acid led to quantitative recovery of bile pigments, which as in the absence of pyridine, comprised a mixture of biliverdin and bilipurpurin. A number of trials were now made in an attempt to convert all the ferrihaem into 'verdohaematin', following the change by measurement of red absorption and diminution of the Soret band. Unfortunately, it was found that, irrespective of the conditions of time, pH or ascorbic acid concentration, the 'verdohaematin' concentration could be increased only slightly over that achieved in earlier experiments. Optical density in the Soret region progressively declined, but when approximately 60% of haematin had been disrupted, further prolonged treatment with ascorbic acid and oxygen led to decrease in red absorption. It appeared impossible, therefore, to achieve complete conversion of haematin into 'verdohaematin' in this system.

Further information on the haematin reaction products was sought through coupling experiments with native human globin. Haematin:ascorbic acid reaction products containing an optimum yield of 'verdohaematin' as judged by increased optical density in the red region of the spectrum were treated with native human globin prepared by the method of Anson & Mirsky (1930) as modified by Jope, Jope & O'Brien (1949). Haematin reaction products (11 ml., equivalent to 86 μg. biliverdin) were incubated at 37° with 11·5 ml. solution containing 126 mg. native globin. The absorption spectrum of the final solution exhibited a plateau in the range of 660–670 mμ. (Fig. 4). This solution (4 ml.) was saturated in the spectrophotometer cell with carbon monoxide, when the maximal absorption was displaced to 620 mμ. The solution was rendered alkaline by addition of 0·4 ml. 20% sodium hydroxide solution and reduction effected by a few fine granules of sodium hydrosulphite. The cell was stoppered, inverted twice and the absorption in the range 600–700 mμ. immediately measured. A minimum quantity of sodium hydrosulphite, saturation with carbon monoxide and rapid optical measurement precluded peroxidative

effects of alkaline hydrosulphite on haemoglobin derivatives derived from residual haematin. A broad maximum was observed at 620–630 mμ. These spectroscopic changes were reminiscent of the behaviour of choleglobin (Lemberg, Legge & Lockwood, 1941*b*). Ferricholeglobin exhibited an absorption band at 670 mμ., which on treatment with coal gas was displaced to 628 mμ. due to carboxycholeglobin. Further treatment with sodium hydroxide and sodium hydrosulphite led to the formation of denatured globin carboxycholehaemochromogen with peak absorption still at 628 mμ., but with increased optical density.

In contrast, verdohaematin combined with native globin to give olive-green verdohaemiglobin with indistinct absorption in the red, reduced by hydrosulphite to verdohaemoglobin which had a sharp band at 665 mμ. (Lemberg & Legge, 1949). Treatment of these compounds with alkaline hydrosulphite produced a yellow pigment and no evidence of conversion into cholehaemochromogen, which absorbs light at 616–618 mμ. (Lemberg *et al.* 1941*a*).

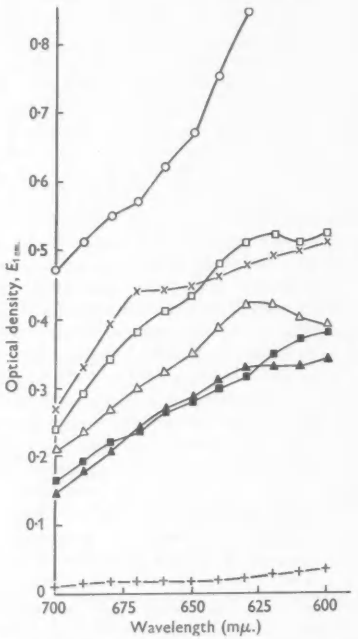

Fig. 4. Reaction of human globin with a product of the haematin-ascorbic acid reaction. ○—○, 14 mg. haematin incubated at 37° for 120 min. with 100 mg. ascorbic acid + O_2. ×—×, 11 ml. final haematin solution (≡ 86 μg. biliverdin) incubated at 37° with 11·5 ml. native human globin solution containing 126 mg. protein. □—□, haematin reaction products-globin saturated with CO. △—△, haematin reaction products-globin + CO + $Na_2S_2O_4$ + NaOH. ■—■, haematin reaction products (5 ml. contains 43 μg. biliverdin) coupled with 100 mg. denatured human globin, and saturated with CO. ▲—▲, haematin reaction products—denatured globin + CO + $Na_2S_2O_4$ + NaOH. +—+, native globin solution, as used for coupling with haematin reaction products, incubated alone at 37°.

When the main bulk of the solution of haematin reaction products which had been coupled with globin was subjected to treatment with 66 % acetic acid in the usual way, no trace of bile pigment could be recovered, in contrast to the liberation of 86 μg. biliverdin before coupling with globin. This behaviour was also that of choleglobin, rather than of a verdohaem derivative (Lemberg & Purdom, 1949).

A similar experiment was made with denatured human globin, in which the pH of the globin solution before addition to the haematin reaction products was necessarily raised to keep the protein dissolved. The spectrum of the solution remained, however, without any localized absorption in the 600–700 mμ. range. The optical density of the native globin solution itself was almost uniformly low in the 600–700 mμ. region of the spectrum.

Experiments with metmyoglobin. Metmyoglobin was prepared as described by George & Stratmann (1952), the final solution in 3 M phosphate buffer, pH 6·8, being dialysed against distilled water, and concentrated at 40° to 0·43 mM, measured as pyridine haemochromogen (Rimington, 1942). The solution exhibited the characteristic absorption bands of metmyoglobin at 630 and 505 mμ. with very weak bands of oxymyoglobin at 583 and 547 mμ. To 50 ml. of 0·43 mM metmyoglobin solution (≡ 14 mg. combined ferrihaem) contained in a Petri dish were added 100 mg. L-ascorbic acid and the mixture was incubated in air at 37° for 2 hr. The reaction was followed spectroscopically using a Beck Hartridge Reversion Spectroscope (Table 1).

Rapid reduction of metmyoglobin occurred, and, after 5 min. incubation, the absorption bands at 634 and 505 mμ. were very weak and diffuse, whilst those of oxymyoglobin at 581 and 547 mμ. were correspondingly intensified. As the reaction proceeded, oxymyoglobin disappeared, and there was progressively increased absorption in the red region of the spectrum, corresponding to the recorded values of 670 and 640 mμ. for cholemyoglobin (Kiese & Kaeske, 1942). There was no evidence of reduced myoglobin with absorption maxima at 595–590 and 560–550 mμ. The red absorption finally extended down to 622 mμ., and was not due to re-formed metmyoglobin, since an absorption band at 504 mμ. was very weak in intensity. Residual unchanged haemoprotein occurred as oxymyoglobin, without autoxidation to metmyoglobin, the concentration of such pigment, however, being greatly diminished as compared with early

Table 1. *Spectroscopic changes in 0·43 mM metmyoglobin solution on incubation with ascorbic acid*

50 ml. 0·43 mM metmyoglobin solution (≡14 mg. ferrihaem) + 100 mg. L-ascorbic acid incubated in air at 37° for 120 min.

Incubation time (min.)	Absorption bands (mμ.)				
0	647–622 (630 Intense)	583. Narrow, very weak	—	505. Wide, intense	433. End of Soret band
5	634. Almost disappeared	581. Sharply defined, intense	547. Sharply defined, intense	505. Weak, diffuse	447. End of Soret band
35	676. Increased absorption 643. Weak 634. Disappeared	582. Almost disappeared	548. Weak	505. Very weak, diffuse	453. End absorption
60	666. Increased absorption 642–620. Diffuse	—	547. Weak	505. Very weak, diffuse	450. End absorption
90	Diffuse absorption in red down to 622	—	546. Weak	505. Very weak	—
120	Diffuse absorption in red down to 622	581. Becoming more intense	545. Becoming more intense	504. Very weak	—

stages of the reaction. The solution remained clear and bright red in colour throughout the experiment, with no evidence of the marked denaturation which obtained with haemoglobin under similar conditions.

Bile pigments were liberated and extracted by the method of Lemberg, Lockwood & Legge (1941), with quantitative determination as fluorescent zinc-bilipurpurin (Kench *et al.* 1950). Biliverdin was the sole product, with yields in four experiments of 4·10, 4·42, 5·54 and 4·47, mean 4·70 mg. This yield (36% theoretical) was the highest observed from any haematin derivative in this series of *in vitro* experiments. A control experiment showed no cholemyoglobin (by acetic acid treatment) nor free bile pigment admixture in the original metmyoglobin solution. Neither autoxidation during preparation nor incubation at 37° in air, therefore, contributed to biliverdin production. George & Stratmann (1952) were also unable to detect either choleglobin or denatured protein during oxidation of myoglobin to metmyoglobin.

Experiments with horse-liver catalase. Horse-liver catalase of Kat. F. 17500 (von Euler & Josephson, 1923), haematin content 0·76% of dry weight, and containing 0·65 mg. combined ferrihaem/ml. solution, was prepared by the method of Keilin & Hartree (1945). 21·6 ml. samples of the final catalase solution were diluted to 50 ml. with distilled water, the total haematin content being 14 mg. The solution was incubated in Petri dishes in air at 37° with addition, in different experiments, of varying quantities of ascorbic acid.

The course of the reaction was followed spectroscopically using a Unicam Quartz Spectrophotometer S.P. 500. Fig. 5 presents data for an experiment in which 100 mg. ascorbic acid were added at the commencement of incubation, 100 mg. after 30 min. and subsequently 50 mg. at 30 min. intervals until 120 min. A generalized increase in spectral absorption was observed, without evidence of localized increased optical density in any region. Subsequent analysis gave a bile pigment yield

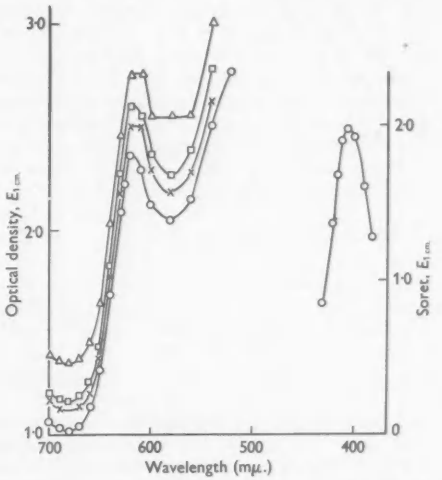

Fig. 5. Changes in absorption spectrum of horse-liver catalase on incubation with ascorbic acid. 50 ml. catalase solution containing 0·28 mg. haematin/ml. (total content 14 mg. combined haematin) were incubated in air at 37°. 100 mg. ascorbic acid added at 30 min. intervals. ○—○, 0 min.; ×—×, 10 min.; □—□, 26 min.; △—△, 87 min. The solution was diluted ×13 for measurement in the Soret region. No increase in bile pigment yield was observed.

equivalent to 22 % of the original haematin content, as compared with 27 % in a control solution, incubated without added ascorbic acid. The negative spectroscopic findings are therefore in conformity with the bile pigment production. In other concurrent experiments, unfortunately not followed spectroscopically, bile pigment formation with yields of 28 and 39 %, respectively, of original haem were found, the latter augmented production arising by treatment of catalase (≡ 7 mg. ferrihaem) with 300 mg. ascorbic acid during 2 hr. incubation, that is, the concentration of ascorbic acid was doubled as compared with the experiment giving least yield of bile pigment. During the course of the experiment a dark greenish brown, floccular precipitate separated, the supernatant remaining dark-green. Biliverdin was the only bile pigment detected among the products derived from horse-liver catalase.

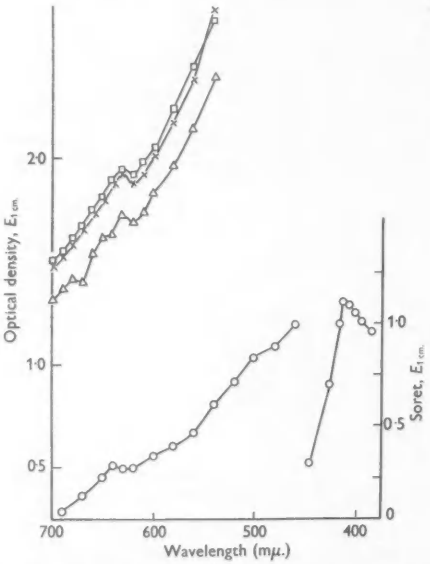

Fig. 6. Changes in the absorption spectrum of horse-radish peroxidase on incubation with ascorbic acid. ○—○, peroxidase solution in 0·25 M phosphate buffer pH 5·6 before addition of ascorbic acid. Spectroscopic readings were made on a solution diluted × 5 with buffer for the visual spectrum, but the data have been calculated on basis of × 4 dilution for comparison with observations made during subsequent incubation with ascorbic acid. 19 ml. peroxidase solution containing 0·26 mg. peroxidase haematin/ml. incubated in air at 37°. 50 mg. ascorbic acid added. For measurement of optical density, 1 ml. reaction mixture diluted × 4 with 0·25 M phosphate buffer pH 5·6. × — ×, 27 min.; □—□, 60 min.; △—△, 120 min. 143 μg. biliverdin were produced.

Experiments with horse-radish peroxidase. Horse-radish peroxidase was prepared from 20 kg. of the root by the method of Keilin & Hartree (1951), and obtained in a solution having Purpurogallin Number 225 (Willstätter & Stoll, 1918), total enzyme units 517, and containing 0·26 mg./ml. peroxidase haem. The solution (1 vol.) was diluted with 0·25 M phosphate buffer pH 5·6 (4 vol.) for measurement of the visual spectrum (Fig. 6). Peroxidase solution (19 ml., equivalent to 0·26 mg. peroxidase haem/ml.) was incubated in a Petri dish in air at 37° for 120 min. following the addition of 50 mg. ascorbic acid. Incubation with ascorbic acid caused a greatly increased general spectral absorption, and the maximal absorption of peroxidase at 640 mμ. was displaced to 630 mμ. A sample of reaction mixture (1 ml.) was removed at 27, 60 and 120 min., and diluted with 3 ml. 0·25 M phosphate buffer, pH 5·6, and the solution centrifuged to remove an interfering brownish precipitate which separated out especially during later stages of the incubation. Very little change in the absorption was observed during the period 27–60 min., but there was a general fall in optical density at 120 min., probably related to the removal of denatured pigmented material which had taken place during this phase of the reaction. The peak at 630 mμ. remained, with an interesting additional localized absorption at 675 mμ. No evidence was obtained of the bands of reduced peroxidase at 594 and 558 mμ.

At the end of the reaction, the products were treated with 66 % acetic acid in the usual way, with liberation of 143 μg. biliverdin, equivalent to 2·9 % original peroxidase haemin. Bilipurpurin could not be detected. No trace of bile pigment could be

Table 2. *Bile pigment formation* in vitro *from haematin and other haem derivatives*

	State of combined iron	Bile pigment yield* (μg.)
Protoporphyrin		0
Ferrohaem	Fe^{2+}	21†
Ferrihaem	Fe^{3+}	385
Haemoglobin	Fe^{2+}	422
Ferrihaemoglobin	Fe^{3+}	532
Metmyoglobin	Fe^{3+}	4700
Ferrohaemalbumin	Fe^{2+}	91†
Ferrihaemalbumin	Fe^{3+}	938
Cytochrome *c*	Fe^{3+}	0
Catalase	Fe^{3+}	1567
Peroxidase	Fe^{3+}	399

* Optimum yield of bile pigments (biliverdin + bilipurpurin) from haem derivative containing 14 mg. haem.

† Ferrohaem and ferrohaemalbumin were prepared from the corresponding ferric compounds by reduction with 0·5 % (w/v) $TiCl_3$. To avoid re-oxidation during the course of the experiment, the reaction time was shortened to 60 min., without supplying extra O_2. Bile pigment yields from ferro-derivatives were slightly less than those from ferric compounds under these conditions.

found when 8 ml. peroxidase solution were incubated in a similar way without addition of ascorbic acid.

The yields of bile pigment derived from metmyoglobin, horse-liver catalase and horse-radish peroxidase are presented for comparison with those obtained from other haem derivatives (Table 2).

DISCUSSION

The primary reaction in the coupled oxidation of oxyhaemoglobin with ascorbic acid has been envisaged by Lemberg & Purdom (1949) as leading to formation of choleglobin by addition of hydrogen peroxide to the α methene linkage, thus lowering the optical density in the Soret region without disrupting the porphyrin system. Further secondary reactions follow, including partial oxidation of the prosthetic group to verdohaem present in verdohaemoglobin, and only the latter type of reaction leads to bile pigment (Callaghan & O'Hagan, 1949; Lemberg & Purdom, 1949). The 10% bile pigment yield on treating the reaction mixture with acid is due to this secondary oxidation having accompanied the primary reaction.

In vitro conversion of haematin into bile pigments has been studied at length, as a simple system likely to provide information of value applying to the breakdown of haemoproteins, in which side reactions were more likely to occur. Spectroscopic examination showed that increased optical density occurred in the red region of the spectrum, and concurrent diminution in the Soret region provided an index of the destruction of the haem. Complete conversion of haematin into bile pigment precursor could not be achieved, and the maximum was reached when 60% of haematin had been disrupted. Addition of pyridine to the final reaction mixture led to the detection therein of verdohaematin, a ready source of bile pigments on treatment with acid.

No bile pigment could be recovered from the haematin reaction products coupled with globin, probably owing to some alteration in the mode of linkage of verdohaematin to globin brought about by acetic acid treatment, leading to a firm bond, resistant to acid and possibly involving the vinyl side chains as suggested by Callaghan (1949) for the similarly resistant cruoralbin. Such changes could account for the non-liberation of bile pigment, but not for the formation of denatured globin-carbon monoxide-cholehaemochromogen, characteristic of cholehaem compounds, which have been shown to possess a closed porphyrin ring (Lemberg & Purdom, 1949). The probable explanation appears to be the presence of both verdohaematin and cholehaematin in the haematin reaction products.

The yield of bile pigment from free haematin in aqueous solution at pH 8·0 was similar to that from oxyhaemoglobin, but the optimum conditions for the conversion differed in that bile pigment formation from oxyhaemoglobin was favoured by low partial pressure of oxygen (Lemberg *et al.* 1941*b*), whilst that from haematin was augmented by vigorous oxygenation and continuous addition of ascorbic acid. As *in vitro* degradation of haematin to bile pigment led to both cholehaematin and verdohaematin, the pathway was probably similar to that of haem combined with protein in oxyhaemoglobin, although here the reactions were complicated by formation of protein derivatives, such as denatured globin cholehaemochromogen ('green pigment').

Regarding the mechanism of bile pigment formation, a coupled oxidation was unlikely to be operative in the case of free haematin, since although ferrohaem was autoxidizable in air, the yield of bile pigment from it was much less than from haematin. No spectroscopic evidence of ferrohaem was obtained during the experiments with aqueous haematin. It was not necessary to assume reduction of haematin in this reaction. Free ferrihaem combined readily with hydrogen peroxide, and behaved as a peroxidase to activate hydrogen peroxide, which brought about the destruction of other haem molecules, or even protoporphyrin (Granick & Gilder, 1947). Autoxidation of ascorbic acid alone must have sufficed to bring about oxidation of haematin. Vigorous oxygenation, continuous addition of ascorbic acid to replace that irreversibly oxidized, and the catalytic effect of haem itself were factors which operated to increase the rate of hydrogen peroxide production. Ferrihaem would combine readily with hydrogen peroxide, and this activated peroxide compound would be responsible for oxidative destruction of more haem molecules. Such a protohaematin peroxide compound has not yet been isolated, although a peroxide complex with free urohaematin I has recently been described by J. Keilin (1952).

Of other haem derivatives studied, only two pigments, protoporphyrin and cytochrome *c*, failed to undergo breakdown to bile pigment in the presence of ascorbic acid and oxygen under the conditions of the experiments (Table 2). The negative result with protoporphyrin confirmed the important role of iron in the reaction with ascorbic acid, and in the autodestruction of haem and its derivatives. All other haem derivatives examined were reactive, and convertible into bile pigment, whether the iron was in the ferrous or ferric condition. The yields of bile pigment from haematin, ferrihaemalbumin and ferrihaemoglobin were all greater than those from the corresponding ferrous compounds, evidence which militates against the ferrous state being an obligatory condition for bile pigment formation.

The optimum conditions for haematin—vigorous oxygenation and continuous addition of ascorbic acid—were most suitable for ferrihaemalbumin, whilst of no advantage compared with incubation in air for methaemoglobin, metmyoglobin and catalase. Under similar conditions to those optimum for conversion of oxyhaemoglobin into choleglobin, metmyoglobin was even more readily disrupted. Myoglobin has been shown to react almost five times more rapidly with oxygen than does haemoglobin (Millikan, 1936), and in consequence of autoxidation is changed almost entirely to metmyoglobin during preparation. Also, in contrast to the slow reduction of methaemoglobin by ascorbic acid *in vitro*, metmyoglobin was observed to undergo almost complete reduction to oxymyoglobin during the first 5 min. incubation. The high yield of bile pigment could be ascribed to increased reactivity with oxygen, associated with minimal denatured by-products.

Catalase and peroxidase were two haemoproteins of particular interest from the point of view of breakdown of their constituent ferrihaem to bile pigment, since they were able to decompose hydrogen peroxide, the active agent in the ascorbic acid reaction. Interaction of hydrogen peroxide with horse-radish peroxidase and with horse-liver catalase leading to formation of complexes has been investigated by Chance (1950) and Keilin & Hartree (1951), and the latter workers have shown that the changes in colour and absorption spectrum of catalase that were brought about by ascorbic acid were due to the formation of a catalase-hydrogen peroxide complex, not to a compound between catalase and ascorbic acid. Horse-liver catalase itself contains a bile pigment haemoprotein in varying proportion (Lemberg, Norrie & Legge, 1939: Sumner, Dounce & Frampton, 1940), which Bonnichsen (1948) has recently shown to arise mainly as an artifact during the course of preparation. Lemberg & Legge (1943) have demonstrated that ascorbic acid can cause inhibition of catalase by accelerating the destruction of the enzyme by added hydrogen peroxide with production of bile pigment haemoprotein. In the present study prolonged incubation of ascorbic acid with horse-liver catalase in one instance augmented bile pigment-haematin from 27 to 39 % of its total haem, and horse-radish peroxidase experienced disruption of its prosthetic group to yield biliverdin to the value of 2·9 % of original haematin content. Considerable denaturation was observed in the peroxidase experiment and also a small localized increase in optical density at 675 mμ.

Cytochrome *c* provided the single negative result for haem–bile pigment conversion in this series of studies of haem derivatives. This might be ascribed to the fact that it was not autoxidizable and did not combine with oxygen. On the other hand, the prosthetic group is bound very firmly to its protein by two thioether linkages through the two side-groups in the 2 and 4 positions, respectively, and it is possible to remove the iron atom without breaking the attachment of haem to protein (Paul, 1951). Thus, it might be possible for reaction with ascorbic acid to occur with oxidation of the haem to form a verdohaemoprotein, which would lose iron readily, but in which the bile pigment-protein linkage would be resistant to acetic acid. In fact, the final absorption spectrum of incubated cytochrome contained, besides the bands of ferro- and ferri-cytochrome *c*, a weak absorption at 623 mμ. (Gardikas & Kench, 1952) not ascribable to any form of cytochrome *c*, and possibly due to formation of a verdohaemochromogen (Bigwood & Thomas, 1940). This 623 mμ. band has been ascribed by Lemberg & Wyndham (1937) to an artifact, probably derived from altered, autoxidizable cytochrome *c*. However, if this absorption were due to such a compound, it proved resistant to acid, and no bile pigments were recovered in any one of several tests.

From these *in vitro* investigations it is clear that the incorporation of the reactive iron atom into the porphyrin ring, whilst conferring diverse biological activity on many haemoproteins, leads to vulnerability of the porphyrin in coupled oxidation with agents, such as ascorbic acid, which liberate hydrogen peroxide. Only cytochrome *c* appeared resistant, and with this exception, there appears no chemical reason why free haematin or any of the haem derivatives here studied, if exposed to the conditions operating in the reticulo-endothelial system in which oxyhaemoglobin is catabolized to bile pigments, should not undergo a similar degradation. Moreover, the present findings concerning the haematin reaction products indicate that linkage with globin does not greatly influence the course of breakdown of the haem prosthetic group. London (1950) has observed that, in the dog, injected [^{15}N]haematin was rapidly converted into stercobilin, whilst in normal man at least 11% of stercobilin was derived from labelled precursors other than the haemoglobin of circulating erythrocytes (London, West, Shemin & Rittenberg, 1950). These authors suggested that myoglobin, or haem not utilized for haemoglobin production could be the source. Similarly, Gray & Neuberger (1952) have postulated that excess of haematin may have been responsible for bile pigment formation in congenital porphyria. Many aspects of the conversion of haematin and haem derivatives into bile pigment both *in vitro* and *in vivo* remain obscure, however, and resolution of these may depend on complementary studies in tissue and enzyme preparations.

SUMMARY

1. Spectroscopic studies have been made on the *in vitro* conversion of haematin and some other haem derivatives into bile pigments through the action of ascorbic acid and oxygen.

2. The conversion of haematin into bile pigments is associated with increased optical density in the range 630–700 mμ. and evidence was obtained for the presence of verdohaematin and cholehaematin in the reaction products.

3. The yield of bile pigment derived from metmyoglobin was approximately eleven times that from oxyhaemoglobin under similar experimental conditions. Cholemyoglobin was observed spectroscopically.

4. Horse-radish peroxidase was converted into bile pigment in 2·9 % yield of its haem content, and the bile pigment haematin content of horse-liver catalase was increased from 27 to 39 %.

5. With the exception of cytochrome *c*, all haem derivatives in the series suffered, in varying degree, oxidative breakdown to bile pigment in the presence of ascorbic acid and oxygen.

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The Measurement of the Components of the Plasminogen–Plasmin System in Biological Fluids

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Streptokinase, a product of haemolytic streptococcal metabolism, is known to activate a serum enzyme precursor, plasminogen, into a proteolytic enzyme, plasmin; plasmin causes the lysis of fibrin and the liquefaction of fibrinous exudate. The action of plasmin can be inhibited by antiplasmin or antiplasmins (nomenclature of Christensen & MacLeod, 1945; Fig. 1). Mullertz & Lasen (1953) have suggested that the activation of plasminogen by streptokinase is not a direct reaction, but is mediated through an intermediary component.

Certain human infections result in the local production of fibrin and fibrinous exudates, which may prove harmful to the patient, and streptokinase has been employed therapeutically in man to disperse such exudates. Fletcher (1954) has shown that the

successful control of this type of therapy requires accurate assay methods for the components of the reaction in biological fluids, and a knowledge of the dynamics of streptokinase activation under physiological conditions. It is the purpose of this communication to describe a suitable method of plasmin assay, assess the accuracy of the method, and investigate some aspects of streptokinase activation by its use.

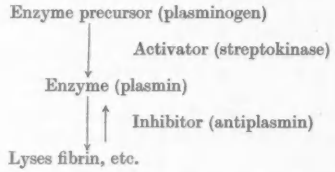

Fig. 1. The plasminogen–plasmin system.

As the aim of these experiments was the control of fibrin lysis in man, fibrin was chosen as the substrate for the measurement of enzymic potency. When the lysis of fibrin is used as an assay method, it is possible to determine either the lowest dilution of enzyme that will lyse a fixed amount of fibrin in a fixed time (Christensen, 1949), or to determine the time which the undiluted enzyme requires to lyse a fixed amount of fibrin (Lewis & Ferguson, 1950). These two methods produce concordant results when using purified reagents, but in biological fluids results are discordant.

The lysis of fibrin clots gives an indeterminate end point, but the method for plasmin assay described here practically eliminates observer error, since the end point is unequivocal.

EXPERIMENTAL

Materials

These were bovine fibrinogen (Armour Laboratories, Chicago), 40% of the total nitrogen being clottable by thrombin; human fibrinogen (Lister Institute, London), 97% of the total nitrogen being clottable by thrombin; thrombin, a single batch of 'Clotting Globulin' (Lederle Laboratories Division, New York); streptokinase–streptodornase (Burroughs Wellcome, Beckenham) and 'Varidase' (Lederle Laboratories Division, New York). Partially purified human plasminogen was prepared by the method of Remmert & Cohen (1949). This material, when freeze-dried and stored at 4°, eventually developed spontaneous plasmin activity. Two batches out of the twenty-three prepared exhibited powerful spontaneous activity, exceeding 15 Christensen units/mg. Chloroform-activated human plasmin was prepared by the method of Rocha e Silva & Rimington (1948). Chloroform-activated bovine plasmin was kindly supplied by Dr E. Loomis (Loomis, George & Ryder, 1947).

Sodium borate buffer, 0·25% (w/v), was used to maintain the pH of the assay reactions at 7·7.

Methods

Plasminogen was assayed by the method of Christensen (1949) with the modifications described by Christensen & Smith (1950).

Units of measurement. The units of streptokinase and plasminogen were those described by Christensen (1949). Plasminogen cannot be measured directly and has to be assayed after conversion into plasmin; this results in the Christensen unit of plasminogen being defined as the enzyme potency required to lyse a fibrin clot derived from a 0·2% (w/v) solution of bovine fibrinogen in a volume of 1 ml. at 35° in 30 min. Such a definition assumes, with the favourable experimental conditions laid down, complete conversion of plasminogen into plasmin to an extent of 1 unit/ml. It is not possible to obtain direct experimental proof of this assumption, and subsequent argument is based on the fact that, though owing to inefficient precursor conversion the Christensen method of plasminogen assay may give too low a reading for the plasminogen content, it will not give too high a reading.

It is, therefore, necessary to have a reference standard of enzyme activity, and this was provided by using a single batch of freeze-dried, chloroform-activated bovine plasmin (Loomis *et al.* 1947). The Loomis preparation assayed at 0·41 mg./ml. was equivalent to 1 Christensen unit of plasmin (mean of twenty determinations, S.D. 6·4%). The unit of plasmin activity is equivalent to the lysis of 0·5 mg. of pure human fibrin in a volume of 1 ml. at 35° in 30 min. The unit of antiplasmin is that amount of inhibitor neutralizing 1 unit of plasmin activity after 10 min. at 35°.

Plasmin assay. Plasmin was assayed as follows: stoppered tubes 7·5 × 1 cm. were placed in a rack inclined at an angle of 25° to the horizontal, immersed in a water bath, thermostatically controlled at 37°. A volume of 0·7 ml. of the solution to be assayed was placed in each tube and allowed to attain the temperature of the bath. Thrombin solution, 0·1 ml. (of sufficient strength to cause complete clotting of the added fibrinogen in 1 min.), was placed in each tube and the requisite quantity of fibrinogen added in a volume of 0·25 ml. The contents of the tubes were mixed by inversion, the time being taken at the instant of adding the fibrinogen. The tubes were replaced in the rack and when, after 1 min., clotting had occurred, a small glass bead (range 140–160 mg.) was placed on the top of the clot and the tube stoppered.

A shaking motor was then started, which rocked the tubes between angles of 25 and 5° from the horizontal 50 times/min. The time of lysis was taken to be when the ball reached the bottom of the tube, and this resulted in a very sharp end point. The shaking motion was required to prevent the ball adhering to the side of the tube. The reciprocal of the lysis time was compared with that produced by a standard solution of the enzyme, and the potency of the unknown solution was read off from the standard graph (Table 1). The reproducibility of the method was approximately ±10% over the range of potencies assayed.

The antiplasmin content of a fluid was ascertained by mixing the fluid with spontaneously active plasmin or chloroform-activated plasmin, incubating the mixture for 15 min. at 37°, and then assaying to determine the loss of activity. The time of incubation was chosen as 15 min., since experiments showed that 95% of the antiplasmin activity would be assayed at the end of this period.

Table 1. *Details of the results for the three fibrinogen concentrations employed for the plasmin assay*

Final fibrinogen concentration	0·125%	0·25%	0·5%
Formula of calculated line for reference preparations	$y=0{\cdot}0647x+0{\cdot}06$	$y=0{\cdot}0357x+0{\cdot}06$	$y=0{\cdot}0255x+0{\cdot}034$
Correlation coefficient	0·971	0·964	0·948
S.D. about regression line	0·0189	0·0192	0·0203
95% confidence limits of potency estimate at mid-point (6-point assay): $\frac{2s}{\bar{x}b\sqrt{n}}$ *	±10·6%	±9·7%	±8·7%
Range: mg. of Loomis enzyme/ml.	0·5–4	1·2–7·5	4–11
Range: Christensen units/ml.	1·25–10	3–19	10–28

Ordinate (y) $1/t$ (min.). Abscissae (x) mg. of Loomis enzyme/ml.

* Approximate formula for linear dose response lines. 6-Point assay refers to six assays of unknown substance. s=standard deviation about regression line; b=slope of line for reference preparation; $\bar{x}$=mean value of determinations; n=number of determinations.

Accuracy of plasmin assay. In order to test the accuracy and reproducibility of the plasmin assay method, weighed quantities of the freeze-dried, chloroform-activated, bovine enzyme (Loomis preparation, 0·41 mg./ml.≡1 Christensen unit) were assayed against the three batches of fibrinogen used over a period of 1 year. All determinations were combined in an attempt to find the maximum variation of the method during this period. Lysis-time readings were in all cases restricted to between 3 and 10 min. and if lysis times greater or less than this period were obtained, a shift was made to another fibrinogen concentration. The results, with the 95% confidence limits, are shown in Table 1.

It was concluded that this method of assay gave reproducible estimates of enzymic potency throughout the course of the investigation. It should be emphasized that the 95% confidence limits given in Table 1 are maximum errors, since with single batches of fibrinogen on a single day the variation about the assay line was less than half that indicated in Table 1. Since each individual experiment was performed under the latter conditions, the lesser error would apply, whilst for comparison between experiments the greater error would be the appropriate measure of accuracy.

To test the applicability of this method to human material the following experiments were performed. Chloroform-activated and spontaneously active human plasmin were diluted, and the diluted solutions assayed for enzyme potency. The enzyme potencies found were plotted against the concentration of the solution and the regression coefficient was calculated. Technical limitations rendered it impossible to use the 0·5% fibrinogen line, but when the regression coefficient was calculated for the chloroform-activated preparation using the other two lines, it was 0·9; the regression coefficient for the spontaneously active preparation was 0·93.

A purified streptokinase-activated plasmin was employed to cover all the assay lines in a similar fashion; the regression coefficient in this case was 0·9 and the standard deviation about the regression line was 12. Since the original concentration was, for convenience, taken as 100, this made the S.D. 12%. The reason for this greater degree of variation was that the assay lines do not superimpose at their junction; when the S.D. of the regression coefficient was calculated for the 0·25% line, it was 6%.

It was concluded that the method of assay was suitable for use with human plasmin solutions, which could be estimated with the same accuracy as bovine preparations.

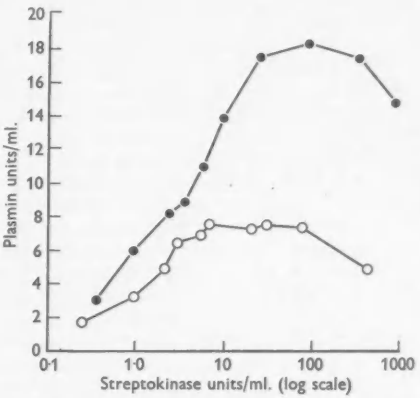

Fig. 2. Plasmin activity (units/ml.) found when differing concentrations of streptokinase are added to two concentrations of plasminogen. ●—●, 30 units plasminogen/ml.; ○—○, 10 units plasminogen/ml.

RESULTS

The dynamics of streptokinase activation

The use of partially purified human plasminogen (8000 units/mg. nitrogen), practically devoid of antiplasmin activity, has allowed the dynamics of plasminogen activation by streptokinase to be studied. Fig. 2 shows the amount of plasmin produced when varying concentrations of streptokinase are allowed to act upon fixed amounts of plasminogen. Small concentrations of streptokinase induce an increase in plasmin activity approximately related to the $\log_{10}$ of the streptokinase concentration employed. Eventually a more or less constant enzymic activity is reached, and no further increase of activity can be produced by increasing the streptokinase concentration as much as tenfold. Concentrations of streptokinase some 20–50 times those inducing maximal enzymic

activity cause a fall in enzymic activity. The explanation of this impairment of enzyme activity is not clear; neither the antiplasmin activity of the streptokinase nor the decay curves of enzyme activated with high streptokinase concentrations will account for this phenomenon. A wide range of streptokinase concentrations can be employed to induce the maximal enzyme activity of a solution, and titrations to ascertain this activity can be performed easily.

The activity produced by fixed concentrations of streptokinase acting upon varying amounts of plasminogen is shown in Fig. 3. Concentrations of streptokinase of 1, 10, and 100 units/ml. were employed in this experiment, the concentrations of plasminogen varied from 1 to 250 units/ml. It is seen that though, with a fixed streptokinase concentration, rising concentrations of precursor result at first in increasing levels of enzymic activity, they eventually fail to give this increase. Considerable conversion of precursor into enzyme is only achieved where the concentration of precursor is low.

An experiment of this nature bears on the validity of the Christensen method of plasminogen assay, which requires that plasminogen should be quantitatively converted into plasmin to the extent of 1 unit/ml. Table 2 shows the enzyme activity produced when low plasminogen concentrations are treated with 100 units of streptokinase/ml. (concentration used for plasminogen assay). It is seen that the enzymic activity produced is approximately proportional to the plasminogen concentration up to 4 units of plasminogen/ml. This finding suggests that the plasminogen assay gives results of good relative accuracy, even though the absolute accuracy remains uncertain.

In order to ascertain the degree of precursor conversion when the optimum rather than a fixed streptokinase concentration was used, the following experiment was performed. Diluted samples of a purified plasminogen solution were individually assayed for their plasminogen content. Each diluted solution was activated with several concentrations of streptokinase, and using the optimum streptokinase concentration ascertained by this procedure, the maximal enzyme concentration for each dilution was determined. This experiment clearly shows (Fig. 4) that it is not possible to activate all the precursor to form the enzyme with streptokinase except under conditions of very low precursor concentration. Streptokinase converts a high proportion of plasminogen into plasmin at low plasminogen concentrations but proves less effective at high plasminogen concentrations. This finding demonstrates the hazard of using any method of plasminogen assay unless the plasminogen concentration is controlled at a low level, as in the Christensen assay.

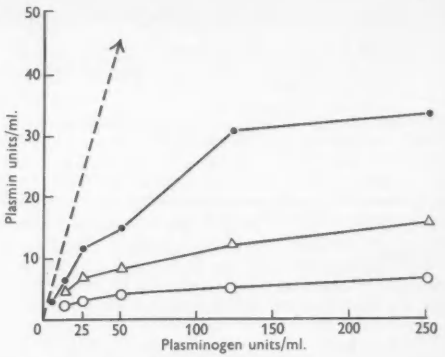

Fig. 3. Plasmin activity (units/ml.) found with differing plasminogen concentrations and three concentrations of streptokinase. ●—●, 100 units streptokinase/ml.; △—△, 10 units streptokinase/ml.; ○—○, 1 unit streptokinase/ml., – – –, theoretical line of 100% conversion of plasminogen into plasmin.

Table 2. *Enzymic activity produced when low concentrations of plasminogen are treated with* 100 *units of streptokinase/ml.*

	Amount of material (units/ml.)						
Plasminogen	0·5	1·0	1·5	2·5	4·0	8·0	12
Plasmin	0·5	1·0	1·5	2·2	3·6	4·5	5·4

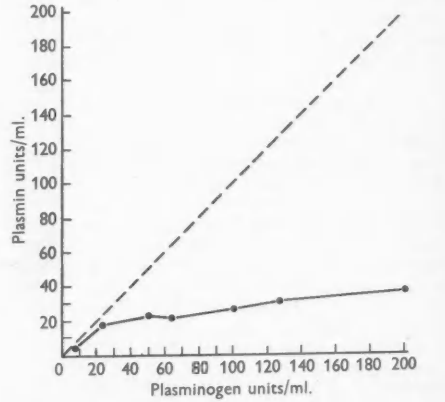

Fig. 4. Conversion of plasminogen into plasmin with 'optimum' streptokinase concentration. 'Optimum' streptokinase concentration is that concentration of streptokinase which will produce maximum fibrinolytic activity in a solution of plasminogen. – – –, theoretical line of 100% conversion of plasminogen into plasmin; ●—●, actual conversion of plasminogen into plasmin under the conditions of the experiment.

Antiplasmin and the properties of the enzyme–inhibitor complex

Plasmin mixed with serum, cerebrospinal or some other body fluids, shows a marked reduction in activity amounting in some cases to complete extinction of activity. This plasmin–antiplasmin complex has properties which influence both the therapeutic potentialities of the enzyme and the assay of enzymic potency in biological fluids.

The experiments of Schulman (1952), using radioactive fibrinogen as a substrate for the measurement of bovine plasmin activity, have shown convincingly that where serum dilutions of the order 1:1000 are used, plasmin forms a stoicheiometric and irreversibly bound compound with its inhibitor.

Fig. 5 shows that when human serum is assayed for antiplasmin activity, using spontaneously active human plasmin as a source of enzyme, the inhibitory effect of the serum is proportional to its concentration. Since the plasmin activity of spontaneously activated preparations of plasminogen is low, it has only been possible to investigate low serum concentrations by this method. The type of inhibition seen in this experiment differs from that described by Schulman in that the plasmin–antiplasmin complex can be partly dissociated by dilution and change in pH as is shown by the following experiment.

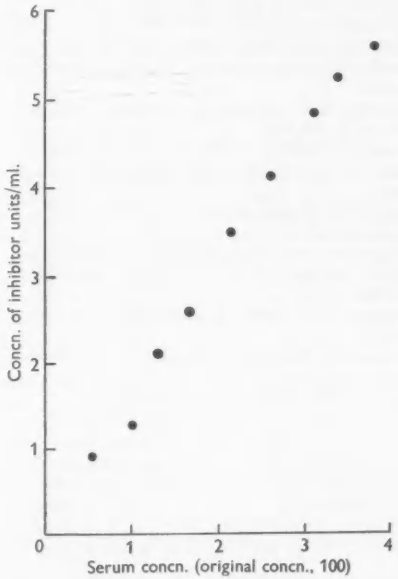

Fig. 5. Antiplasmin activity (inhibitor units/ml.) of varying concentrations of human serum.

Spontaneously active human plasmin (activity 23 units/ml.) was neutralized by a 1:10 dilution of human serum. The mixture was shown to be devoid of plasmin activity, the pH was altered to 5·3 by 1% (w/v) acetic acid, the mixture cooled, and the precipitate spun down. On being made up to the original volume, the precipitate contained 40% of the original enzymic potency.

Certain aspects of plasmin inhibition by solutions of high protein content cannot be accounted for by specific and irreversible combination of plasmin with an inhibitor. Macfarlane & Biggs (1946) demonstrated that the physiological fibrinolysis induced in man by fear or anxiety could only be demonstrated after considerable dilution of the plasma, and this observation has been repeatedly confirmed. Similarly, when solutions of spontaneously active human plasmin are mixed with sufficient serum to extinguish all fibrinolytic activity, some activity will be present on dilution of the solution. These findings are inconsistent with the theory of an irreversible and stoicheiometric combination of enzyme and inhibitor, and strongly suggest that a further mechanism is responsible for much of the plasmin inhibition shown by solutions of high protein content.

The fact that an easily dissociable, non-specific inhibition rather than a specific inhibition of plasmin is important at high protein concentrations may be seen from the following experiment. A solution of spontaneously active plasmin with a concentration of 10 mg./ml. was serially diluted with borate buffer and assays were performed on the dilutions. The effects of specific inhibition can be

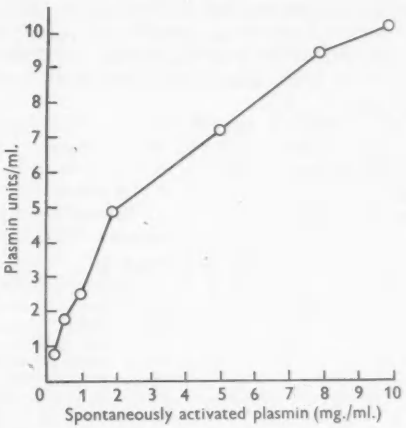

Fig. 6. Plasmin activity units/ml. produced by varying concentrations of 'spontaneously' activated human plasmin. The enzyme activity is not proportional to the concentration of dissolved material.

disregarded in such an experiment since, in view of the high excess of enzyme in all dilutions, the demand of a specific inhibitor would be satisfied. The results are shown in Fig. 6, where it is seen that the enzymic activity is not proportional to the concentration of dissolved plasmin, this activity being less than would be expected where the concentration of dissolved plasmin is high.

The finding that non-specific inhibition of plasmin is important in the inhibition of plasmin by body fluids means that the assay of active enzyme in biological fluids must be performed at the actual concentration in which the enzyme is present, otherwise the values obtained will be too high.

DISCUSSION

The experimental findings concerning the degree of conversion of precursor into enzyme by streptokinase at various precursor concentrations indicate the necessity of assaying plasminogen under conditions causing a high degree of conversion of precursor into enzyme. These conditions require a high dilution of precursor, such as is used in the Christensen method of assay. The difficulty of converting high plasminogen concentrations into equivalent amounts of enzyme with streptokinase has important implications with regard to the therapeutic uses of streptokinase.

The demonstration that non-specific inhibition of plasmin is important at high protein concentrations shows that the true level of fibrinolytic enzyme can only be ascertained when assays are conducted in biological fluids as near the original concentration as possible. This precaution is doubly necessary when assaying enzyme activity induced by streptokinase. Streptokinase-containing solutions will alter their content of free fibrinolytic enzyme on dilution, and errors from this source cannot be easily corrected.

The degree of availability for physiological purposes of enzyme that has undergone non-specific, rather than specific, inhibition in the body must be regarded as uncertain. The demonstration that pH changes may affect the dissociation of the enzyme–inhibitor complex suggests that minor local changes *in vivo* may cause the release of enzyme. *In vitro*, enzyme that has undergone non-specific inhibition is incapable of lysing fibrin. The time of lysis method of determination, as developed in this communication, measures only free fibrinolytic enzyme, and this method is therefore suitable for determining the enzymic potency of biological fluids during the course of therapy with fibrinolytic substances.

SUMMARY

1. A method of fibrin lysis with an objective end point, and suitable for the measurement of free-plasmin activity in biological fluids is presented. The accuracy of the method over the range 1–28 Christensen units/ml. is ± 10 %.

2. Streptokinase converts a high proportion of plasminogen into plasmin at low plasminogen concentrations, but converts only a low proportion at high plasminogen concentrations.

3. The important role played by non-specific inhibition of plasmin as against specific inhibition of plasmin in biological fluids has been emphasized, and the possibility that such enzyme may be physiologically active has been suggested.

I am indebted to Prof. Sir Alexander Fleming and Prof. R. Cruickshank for much encouragement and advice. I wish to thank Dr E. Loomis of Parke Davis Laboratories for a generous gift of chloroform-activated bovine plasmin, and Dr P. Armitage of the Medical Research Council Statistical Unit for suggesting a method of analysing the results.

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Derivatives and Kinetics of Lactoperoxidase

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Following the investigations of Elliott (1932*a*, *b*) and Yakashuji (1939) on the peroxidase occurring in cow's milk, Theorell & Åkeson (1943) showed that this enzyme differs in its prosthetic group from both myeloperoxidase and the known plant peroxidases. Chance (1949, 1950) has studied the kinetics of lactoperoxidase. The present paper describes the absorption spectra of various derivatives of lactoperoxidase and an investigation into some aspects of the kinetics of the enzyme.

For the purpose of the present study a method of obtaining the enzyme in high spectroscopic purity was required. Since the method used by Theorell and co-workers (Theorell & Åkeson, 1943; Theorell & Pedersen, 1944), who obtained homogeneous crystalline preparations of the enzyme, was not published in detail, and moreover required the use of electrophoresis, a suitable method was developed and is briefly described in this paper. However, this method gives a non-crystalline preparation of 40–50% purity. After the completion of this work a paper has appeared by Polis & Shmukler (1953) describing a chromatographic method by which they prepared the pure crystalline enzyme.

EXPERIMENTAL

Measurement of lactoperoxidase activity

Chance (1950) has shown that the modified Willstätter method of measuring lactoperoxidase activity used by Theorell & Åkeson (1943) gave low purpurogallin numbers owing to the low percentage saturation of the enzyme with peroxide. Chance (1949, 1950) has investigated the kinetics of lactoperoxidase action spectrophotometrically using reduced cytochrome *c*, hydroquinone, ascorbic acid, guaiacol and pyrogallol as hydrogen donors. In the present work the peroxidation of pyrogallol has been measured spectrophotometrically under conditions such that the reaction of the enzyme with its substrate (H_2O_2) was limiting. Because it was not desirable to saturate the enzyme with peroxide (see later) it was necessary to use the same initial peroxide concentration for all measurements.

In Table 1 are listed the reagents used in the activity test, together with their concentrations and in the order in which they were added to a 1 cm. silica cell. A stop-watch was started immediately after the addition and mixing of the peroxide. Readings of optical density at 300 mμ. were recorded against time for 1–2 min. using a Beckman Model DU Spectrophotometer. The reference cell contained distilled water. The increase in optical density/min. was determined from the slope of the straight line obtained by plotting the readings against time. The measurements were corrected for a slight 'blank' which was determined separately by substituting water for the enzyme in the test. All reaction rates have been corrected to 20°, assuming the rate of reaction to be increased by a factor of 2·0 for an increase in temperature of 10°.

The method gave good proportionality between enzyme concentration and increase in optical density at 300 mμ./min.

Enzyme activity unit. A unit of enzyme activity has been defined so that 1 unit/ml. in the activity test gives an increase in optical density at 300 mμ. of 10/min. The specific activity (S.A.) of an enzyme preparation is defined as the number of units of activity per mg. dry wt. or per mg. protein weight of enzyme preparation. The protein content was determined spectrophotometrically at 280 mμ. from the expression, concentration of protein (mg./ml.) = 1·43 × optical density at 280 mμ. (1 cm. cell). The factor 1·43 was determined experimentally, using an impure enzyme preparation.

Partial purification of lactoperoxidase

Methods of estimating enzyme purity. The purity of the enzyme was measured by two methods: (1) determination of specific activity, and (2) measurement of the haem

Table 1. *Reagents for test of enzyme activity*

For conditions see text.

Reagent	Concn. (M)	Vol. added (ml.)	Final concn. (M)
Pyrogallol*	6×10^{-2}	0·20	4×10^{-3}
Phosphate buffer, pH 6·5	0·1	1·00	$3{\cdot}3 \times 10^{-2}$
Enzyme solution†	—	0·10	—
Distilled water	—	1·40	—
H_2O_2‡	$4{\cdot}36 \times 10^{-3}$	0·30	$4{\cdot}36 \times 10^{-4}$

* Pyrogallol—purified by sublimation under reduced pressure—was supplied by Dr E. F. Hartree. A fresh solution was made in 0·05 M phosphate buffer, pH 6·5, for each series of determinations. Tests showed that after 5 hr. autoxidation at room temperature and pH 6·5 no enzyme-inhibitory substances were produced.

† Enzyme—diluted at 0° in 0·05 M phosphate buffer, pH 6·5, immediately before use so that 0·10 ml. gave an increase in optical density at 300 mμ. not greater than 0·2/min.

‡ Hydrogen peroxide—freshly diluted from stock solution of approx. 4×10^{-2} M, kept at 4°, and titrated against standardized permanganate before the appropriate dilution.

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Table 2. *Enzyme purification*

Stage	Milk	1	2*A*	2*B*	3	4	5*A*	5*B*	5*C*
Activity units	31 700	20 600	5450	2000	6000	2850	520	960	99
Dry weight (D.W.) or protein wt. (P.W.) (mg.)	$6{\cdot}3\times10^6$ (D.W.)	$2{\cdot}6\times10^5$ (P.W.)	—	—	$1{\cdot}3\times10^4$ (P.W.)	2000 (P.W.)	344 (P.W.)	228 (P.W.)	31 (P.W.)
Specific activity	0·005	0·08	—	—	0·47	1·42	1·51	4·2	3·18
Original activity units (%)	100	65	17·3	6·3	19	9·1	1·6	3·0	—
Enzyme (mg.)*	3740	2430	—	—	708	336	61	113	12
Purity (%)†	—	—	—	—	5·5	14·6	15·4	49·6	60·1
$\frac{E\,412\ m\mu.‡}{E\,480\ m\mu.}$	—	—	—	—	7·7	—	8·8	10·0	8·6
$\frac{\text{Purity (\%)†}}{\text{Specific activity}}$	—	—	—	—	11·7	10·3	10·2	11·8	18·9

* Calculated as activity units/8·47, see text.
† Spectrophotometric measurement, method (2), see text.
‡ Measure of contamination by red pigment. For pure enzyme, ratio = 10·7, see text.

absorption (Soret band at 412 mμ.) relative to that of the protein (280 mμ.). In this method the percentage purity of the enzyme is defined by the expression, 1·11 × optical density at 412 mμ. × 100/optical density at 280 mμ., where 1·11 is the ratio of the optical density at 280 mμ. to that at 412 mμ. calculated for the pure enzyme from the data of Polis & Shmukler (1953). The method has been used to detect the formation of soluble, inactivated enzyme. The presence of inactivated enzyme is shown by an increase in the ratio, percentage purity (method 2)/specific activity above the value 100/8·47 = 11·8 (see fraction 5*C*, Table 2).

The specific activity for the pure enzyme was calculated by extrapolation using a preparation in which the specific activity was proportional to the percentage purity (method 2) over a wide range of purification. This value was found to be 8·47. The number of activity units divided by 8·47 thus gives the approximate weight (mg.) of enzyme in any preparation.

The separation of the enzyme from a red pigment having a broad band at 480 mμ. (Theorell & Pedersen, 1944) and no Soret band (Polis & Shmukler, 1953) was followed by comparing the ratio, optical density at 412 mμ./optical density at 480 mμ., found for the lactoperoxidase preparation, with that calculated for the pure enzyme from the data of Polis & Shmukler (1953).

Method of purification. In view of the detailed publication by Polis & Shmukler (1953) of a method by which the crystalline enzyme can be prepared, only a brief description of the method used in the present work is given. The results of this method are tabulated in Table 2.

Stage 1. Separated milk (70 l.) was fractionated in two lots approximately according to the procedure of Elliott (1932*a*) and the resultant enzyme solutions were combined (vol., 2 l.).

Stage 2*A*. Further procedures were carried out in the cold where possible. Thoroughly dialysed stage-1 enzyme solution was treated at pH 5·5 with 3 l. of tricalcium phosphate gel (50 mg./ml. prepared according to Keilin & Hartree, 1951) to adsorb protein impurities. More protein impurities were removed after precipitation at pH 7·7. The enzyme was concentrated by $(NH_4)_2SO_4$ precipitation.

Stage 2*B*. Enzyme was recovered from the phosphate gel centrifugate of the previous step by elution with 3 l. of 1·5% (w/v) $(NH_4)_2SO_4$ soln. (pH 5·5–6·0) and collected after precipitation with $(NH_4)_2SO_4$.

Stage 3. Dialysed stages-2*B* and -2*A* enzyme solutions were combined and the enzyme was precipitated by $(NH_4)_2SO_4$ (70% saturation). Vol., 410 ml.

Stage 4. The product of stage 3 was further purified by treatment with tricalcium phosphate gel (400 ml., 50 mg./ml.) as in step 2*A*, the enzyme collected from the supernatant by $(NH_4)_2SO_4$ precipitation and dialysed. Enzyme was recovered from the phosphate gel as in step 2*B*, further treated with gel to give approximately the same purity (14·5%) as the first supernatant of this step and concentrated by $(NH_4)_2SO_4$ precipitation. The two products were then combined.

Stage 5. Stage-4 enzyme was fractionated with $(NH_4)_2SO_4$ at pH 7·7 and the fraction collected between the limits 48–55% (w/v) was dialysed and again treated with tricalcium phosphate gel. Fractionation of the gel supernatant with $(NH_4)_2SO_4$ (pH 7·7) between the limits 45–50, 50–56, 56–64% (w/v) gave three fractions which were labelled stages 5*A*, 5*B* and 5*C*, respectively. Because of the satisfactory spectroscopic purity of the stage-5*B* preparation and because of the low yields, further purification was not sought. Under the conditions of purification employed the enzyme was found to be very labile above the level 40–50% purity and in further purification the ratio, percentage purity/specific activity, rose (see Table 2, fraction 5*C*).

RESULTS

Theorell & Åkeson (1943) found that lactoperoxidase was inactivated by high concentrations of hydrogen peroxide. The concentration of hydrogen peroxide in the activity test described in the present paper is below that which causes significant inactivation of the enzyme under the conditions of the test. In Fig. 1 is shown the effect of a range of peroxide concentration on a purified enzyme pre-

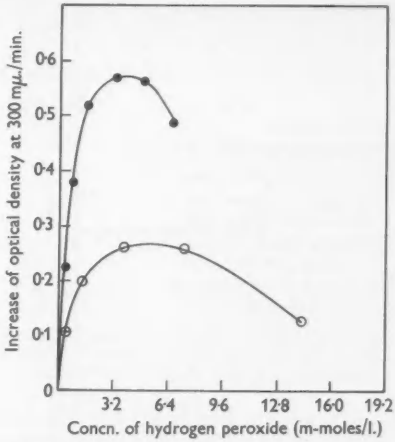

Fig. 1. Effect of peroxide concentration on lactoperoxidase activity using 60% pure enzyme (○) and crude (●) (<1% pure) enzyme fraction. At high concentrations of peroxide where density/time plot is not a straight line, the rate of increase of optical density at 300 mμ. was calculated from the first two readings made within 30 sec.

paration and a crude enzyme preparation. Using a spectrophotometric method of measuring the oxidation of dihydroxyphenylalanine (dopa), Polis & Shmukler (1953) found that lactoperoxidase is inhibited by concentrations of hydrogen peroxide above about 1×10^{-7} M. It is shown by these authors that peroxide competes with dopa for a 'reactive site' on the enzyme. This competitive inhibition was not evident in the present study with pyrogallol as hydrogen donor.

Michaelis constant

The inactivation of the enzyme by peroxide shown in Fig. 1 shows that it is not possible for the purpose of the activity test to saturate the enzyme with peroxide. Such an activity test is only valid therefore for the purpose of following the purification of the enzyme if the Michaelis constant (K_m) remains constant throughout the purification. The K_m for the enzyme-hydrogen peroxide reaction was determined at three stages in the purification of the enzyme (less than 1, 50 and 60% purity) and the values found were $1{\cdot}08 \times 10^{-3}$, $0{\cdot}96 \times 10^{-3}$ and $1{\cdot}2 \times 10^{-3}$ M, respectively, when the concentration of pyrogallol was 4×10^{-3} M. Thus, purification of the enzyme had no significant effect upon K_m. The average value for K_m ($1{\cdot}08 \times 10^{-3}$ M) is used later in this paper as the experimentally determined value. It is shown that this value is in good agreement with the approximate value calculated by Chance. In Fig. 2 the graphic evaluation of K_m is given for the enzyme of 60% purity.

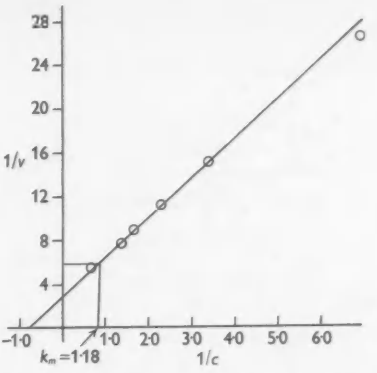

Fig. 2. Graphic evaluation of Michaelis constant for lactoperoxidase (reaction with H_2O_2) using an enzyme preparation of 60% purity. c = millimolar concentration of H_2O_2 and v = velocity in activity test. Results plotted according to method of Lineweaver & Burk (1934).

Absorption spectra of lactoperoxidase and derivatives

The positions of the absorption bands of lactoperoxidase and various derivatives have been given by Yakashuji (1939), Theorell & Åkeson (1943), Theorell & Pedersen (1944), Polis & Shmukler (1953) and Chance (1950). Theorell & Pedersen have given values for the absorption of the electrophoretically homogeneous enzyme at various wavelengths. Chance has given absorption curves for the Soret bands of lactoperoxidase and its compounds formed by hydrogen peroxide. Polis & Shmukler have given absorption curves for the free enzyme and for its compound with excess of hydrogen peroxide.

Absorption curves are given in the present paper for lactoperoxidase (Figs. 3, 4), its cyanide and fluoride derivatives (Fig. 3), and carbon monoxide and pyridine haemochromogen derivatives (Fig. 4). The measurements were made in a Hilger 'Uvispec' Spectrophotometer, the wavelength scale of which was checked using the band of 550 mμ. of reduced cytochrome *c*. The solutions were prepared as shown in Table 3. The absorption curve shown for the free enzyme in Figs. 3 and 4 is similar to that given by Theorell & Pedersen (1944) and Polis & Shmukler (1953). The absorption maxima of the free lactoperoxidase, like those of horse-radish peroxidase, are considerably less sharp than those of the derivatives. Thus lactoperoxidase is also more easily characterized in crude preparations by the preparation of one of these derivatives.

An alkaline form of lactoperoxidase, analogous to that formed from horse-radish peroxidase (Keilin & Hartree, 1951) could not be demonstrated in the present study. With increasing pH the acid

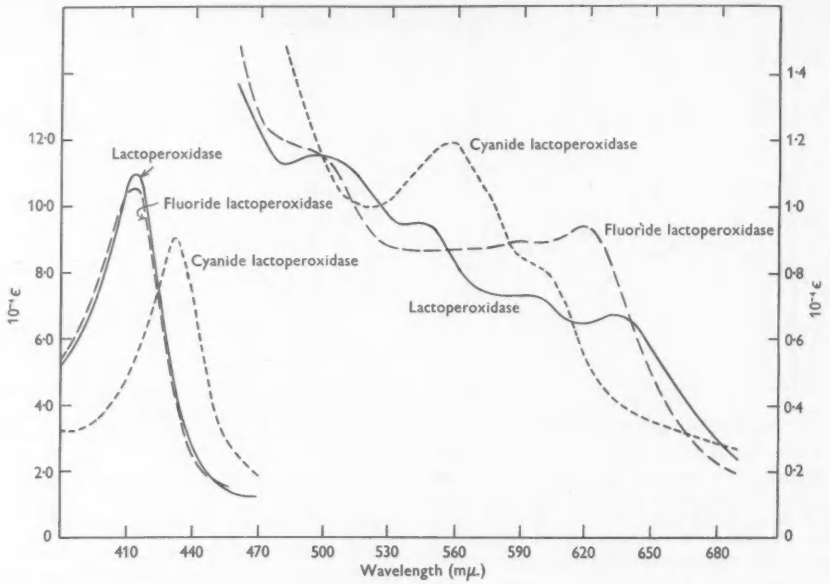

Fig. 3. Absorption spectra of lactoperoxidase and of its cyanide (CN^-) and fluoride (F^-) compounds. For details of solutions used see Table 3. $\epsilon = (\log_{10} I_0/I)/cl$, where c = molar concentration of haematin and l = optical depth in cm.

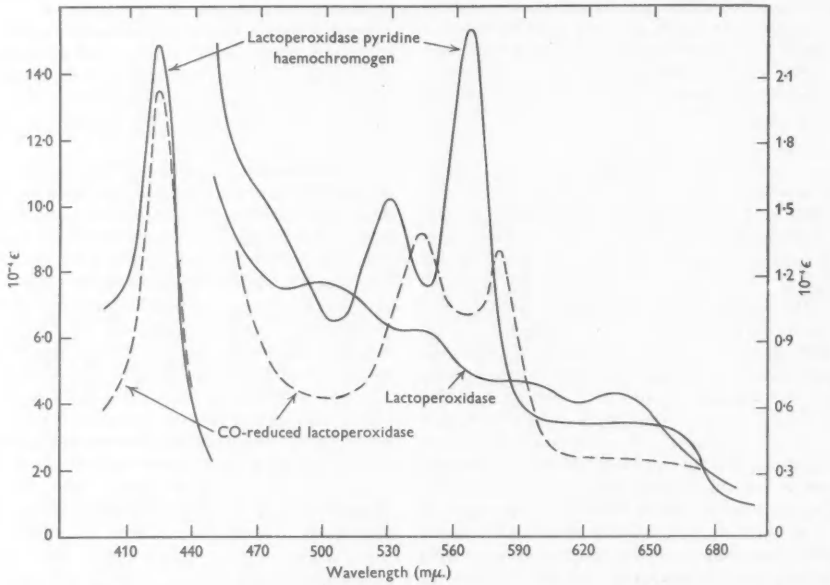

Fig. 4. Absorption spectra of lactoperoxidase, carbon monoxide-reduced lactoperoxidase and the pyridine haemochromogen formed from lactoperoxidase. For details of solutions used see Table 3. For definition of units see Fig. 3.

Table 3. *Preparation of solutions for spectrophotometric measurements on lactoperoxidase and various derivatives*

The mixtures were measured in 1 cm. silica cells. Reference cells contained all reagents except lactoperoxidase.

Enzyme* (ml.)	Additional reagents	Compound formed
1·0	0·2M Phosphate buffer (2·0 ml.) pH 5·6	Lactoperoxidase
1·0	As above, saturated with CO + 20 mg. $Na_2S_2O_4$†	CO-reduced lactoperoxidase
1·0	0·2M Phosphate buffer (2·0 ml.) pH 5·6, containing 0·096M-KCN	Cyanide lactoperoxidase
1·0	0·2M Phosphate buffer (2·0 ml.) pH 5·6, containing 0·63M-NaF	Fluoride lactoperoxidase
1·0	1·25 ml. H_2O + 0·6 ml. pyridine + 0·15 ml. 2·5N-NaOH + 20 mg. $Na_2S_2O_4$†	Pyridine haemochromogen

* 49·6% pure lactoperoxidase—stage-5*B* preparation, $3{\cdot}78 \times 10^{-5}$M. The molarity was calculated from the optical density at 630 mμ. and the data of Theorell & Pedersen (1944).

† Solutions were covered by a layer of paraffin during measurements.

form of lactoperoxidase persisted until, at pH 11·4, a further increase in pH caused the intensity of the Soret band to drop considerably.

DISCUSSION

It is of interest to compare the spectra of lactoperoxidase and its derivatives with those of other haem compounds such as horse-radish peroxidase, catalase and methaemoglobin, absorption curves for which have been given and compared by Keilin & Hartree (1951), and myeloperoxidase, absorption bands for which have been published by Agner (1941). The absorption curves for the first four compounds are of the same general pattern, but there are three important respects in which their spectral properties differ. (1) Of the free haemoproteins only lactoperoxidase has a faint band in the vicinity of 600 mμ. Methaemoglobin has a small maximum at 580 mμ. (2) The general level of absorption/mole haemin varies considerably. In general, over the visible range of the spectrum, the order of intensity is catalase > lactoperoxidase > horse-radish peroxidase and methaemoglobin. (3) Despite the similarity in the general shape of spectra there are significant variations in the positions of absorption bands.

In Table 4, absorption bands for all the above haematin compounds are given for comparison. The absorption bands of myeloperoxidase are quite atypical and, moreover, this enzyme does not react with carbon monoxide or fluoride. According to Foulkes, Lemberg & Purdom (1951), when myeloperoxidase was reduced with dithionite and made alkaline, the type of spectrum did not change. On boiling, however, the bands of protohaemochromogen were obtained. For the other haemoproteins the bands of the cyanide and fluoride derivatives are of particular interest. The α and β bands of catalase and lactoperoxidase cyanide compounds are very similar, and are 12 and 20 mμ. farther to the red compared with the cyanide compounds of methaemoglobin and horse-radish peroxidase, respectively. In the fluoride compounds, however, the bands of the catalase derivative lie farther to the blue whereas the strong band of lactoperoxidase fluoride is approximately 10 mμ. farther to the red compared with the bands for the fluoride compounds of methaemoglobin and horse-radish peroxidase. This comparison of the band positions of the cyanide and fluoride derivatives of catalase with those of the same derivatives of horse-radish peroxidase and methaemoglobin is an outstanding example of the effect of specific proteins on the spectrum of a common prosthetic group.

The fact that the bands of lactoperoxidase derivatives are found consistently at the longer wavelengths is evidence in addition to that supplied by the haemochromogen, that the prosthetic group of lactoperoxidase is not protohaematin. The similarity in the general pattern of the absorption spectra of derivatives of lactoperoxidase and protohaematin enzymes suggests, on the other hand, that lactoperoxidase haematin is not greatly different from protohaematin. The reversion of heated, alkaline, reduced myeloperoxidase to protohaemochromogen strongly suggests that the arrangement of the porphyrin side-chains of this enzyme is similar to, or identical with, that of protoporphyrin. Moreover, myeloperoxidase porphyrin must still contain its four methene carbon atoms one of which, according to Foulkes *et al.* (1951), is probably in an oxidized state.

Kinetics

Theorell & Åkeson (1943) have shown that lactoperoxidase forms two spectroscopically observable compounds with hydrogen peroxide which are analogous to complexes II and III of horse-radish peroxidase. Using his rapid-flow technique, Chance (1950) was able to demonstrate the formation of

Table 4. *Comparison of absorption bands of horse-radish peroxidase* (H.-R.P.), *methaemoglobin* (MetHb), *catalase*, *lactoperoxidase* (L.P.) *and myeloperoxidase* (Myel.P.)*

Compound	Enzyme	Absorption maxima (mμ.)
Free enzyme	H.-R.P.	403, 497, 545, 641
	MetHb	405, 505, 540, 580, 632
	Catalase	407, 500, 540, 627
	L.P.	412, 500, 550, 600, 635
	Myel.P.	430, 495, 570, 625, 690
Reduced enzyme	H.-R.P.	437, 556, 590
	Catalase	Not reducible by $Na_2S_2O_4$
	L.P.	442, 565, 600
	Myel.P.	475, 590, 637
CO-reduced enzyme	H.-R.P.	423, 542, 572
	L.P.	425, 545, 582
	Myel.P.	No reaction
Cyanide enzyme	H.-R.P.	423, 538, 570
	MetHb	415, 543, 578
	Catalase	425, 558, 590
	L.P.	432, 558, 595
	Myel.P.	438, 634
Fluoride enzyme	H.-R.P.	404, 487, 560, 612
	MetHb	405, 488, 550, 608
	Catalase	408, 500, 532, 600
	L.P.	413, 500, 590, 620
	Myel.P.	No reaction
Pyridine haemochromogen	H.-R.P., MetHb, Catalase	Pyridine protohaemochromogen: 525, 558
	L.P.	425, 530, 566
	Myel.P.	Heating with alkaline $Na_2S_2O_4$ gives protohaemochromogen

* Data for first three enzymes derived from absorption curves of Keilin & Hartree (1951), of myeloperoxidase from Agner (1941).

a primary compound which could not be observed by Theorell & Åkeson because of its short life. Chance (1950) has suggested the following mechanism for the action of lactoperoxidase. In these reactions, as rewritten below, the symbol 'FeOH' represents the free enzyme in which the OH group is bound to the iron atom.

$$\underset{(e-p-q)}{\text{FeOH}} + \underset{(s)}{\text{HOOR}} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{(p)}{\text{Fe(OOR)—I}} + \text{H}_2\text{O}, \quad (A)$$

$$\underset{(p)}{\text{Fe(OOR)—I}} \overset{k_7}{\longleftrightarrow} \underset{(q)}{\text{Fe(OOR)—II}}, \quad (B)$$

$$\underset{(q)}{\text{Fe(OOR)—II}} + \underset{(a)}{\text{AH}_2} \overset{k_4 = k_3/[\text{AH}_2]}{\longleftrightarrow} \text{FeOH} + \text{ROH} + \text{A}. \quad (C)$$

Chance (1949, 1950) found that the experimentally determined value of k_7 varied with the concentration of the hydrogen donor, $[AH_2]$. George (1952*a*, *b*) and Chance (1952) have recently produced evidence suggesting that reactions (*B*) and (*C*) should be written,

$$\text{Compound I} + \text{AH}_2 \longleftrightarrow \text{Compound II} + \text{A}. \quad (B')$$

$$\text{Compound II} + \text{AH}_2 \longleftrightarrow \text{Free enzyme} + \text{ROH} + \text{A}. \quad (C')$$

Thus, in reaction (*B*) we may write $k_7 = k_8([AH_2])$.

For the mechanism described in reactions (*A*), (*B*) and (*C*), Chance (1950) found the following experimentally determined values for the reaction constants at 25–30° and pH = 7·0.

$$k_1 = 2{\cdot}2 \times 10^7\ \text{M}^{-1}\ \text{sec.}^{-1};\ k_7 = 4\ \text{sec.}^{-1}$$

(in the absence of added hydrogen donor, AH_2) and k_4 (pyrogallol) $= 6{\cdot}6 \times 10^6\ \text{M}^{-1}$ sec.$^{-1}$. The fact that k_7 does not equal zero in the absence of added donor demonstrates the presence of a donor in the enzyme preparation. In the following derivation it is assumed that the pyrogallol concentration is very much greater than the concentration of endogenous donor and that k_8 is of the same order as k'_8, where the latter is the reaction constant for the endogenous donor.

Derivation of expressions describing Chance's mechanism of action of lactoperoxidase. The concentrations of the components are given in brackets in equations (*A*), (*B*) and (*C*). The total enzyme concentration is *e*.

In the steady state,

$$dp/dt = k_1(e-p-q)s - k_2 p - k_7 p = 0, \quad (1)$$

$$dq/dt = k_7 p - k_4 aq = 0. \quad (2)$$

From (2), $q = k_7 p/k_4 a$. Substituting this value in equation (1),

$$p = \frac{k_1 es}{k_2 + k_7 + k_1 s + k_1 k_7 s/k_4 a}.$$

The velocity of the reaction, $v = k_7 p$, is

$$\frac{k_1 k_7 es}{k_2 + k_7 + k_1 s + k_1 k_7 s/k_4 a}.$$

Since, according to Chance, k_2 is insignificant compared with k_7, this becomes

$$v = \frac{k_1 k_7 es}{k_7 + k_1 s(1 + k_7/k_4 a)}.$$

Dividing numerator and denominator by

$$k_1 s(1 + k_7/k_4 a)$$

$$v = \frac{\dfrac{k_7 e}{1 + k_7/k_4 a}}{1 + \dfrac{1}{s}\left(\dfrac{k_7}{k_1(1 + k_7/k_4 a)}\right)}$$

$$= \frac{V}{1 + K_m/s}, \qquad (3)$$

where V, the velocity at infinite substrate concentration, equals $\dfrac{k_7 e}{1 + k_7/k_4 a}$, and K_m, the Michaelis constant, equals $\dfrac{k_7}{k_1(1 + k_7/k_4 a)}$.

Substituting $k_7 = k_8 a$, we find

$$K_m = \frac{k_8 a}{k_1(1 + k_8/k_4)}. \qquad (4)$$

Michaelis constant. Chance (1950) calculated the K_m for lactoperoxidase ($s = [H_2O_2]$) to be approximately 7×10^{-3} M for a pyrogallol concentration of 20×10^{-3} M. The experimentally determined value of $1{\cdot}08 \times 10^{-3}$ M given in this paper was obtained using a pyrogallol concentration of 4×10^{-3} M. Since, from equation (4), K_m is proportional to the pyrogallol concentration, the experimentally determined value can be multiplied by 5 for comparison with Chance's calculated K_m. The value thus obtained, $5{\cdot}4 \times 10^{-3}$ M, is in reasonable agreement with the approximate value calculated by Chance.

Evaluation of k_8 and k_7. The value of k_8 can be calculated from equation (4) by substitution of numerical values for the other terms as follows: $K_m = 1{\cdot}08 \times 10^{-3}$ M (experimental); $a = 4 \times 10^{-3}$ M (the concentration used in the present study); k_1 and $k_4 = 2{\cdot}2 \times 10^7$ and $6{\cdot}6 \times 10^6$ M^{-1} sec.$^{-1}$, respectively (Chance, 1950). Thus

$$k_8 = 5{\cdot}85 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1}$$

and

$$k_7 = 2{\cdot}37 \times 10^5 \text{ sec.}^{-1}.$$

Chance (1952) found that k_8 for horse-radish peroxidase was greater than k_4, as was to be expected from the earlier finding that compound II was the rate-limiting intermediate. The value of the ratio, k_8/k_4 varied from about 40 for *p*-aminobenzoic acid as donor to about 80 for nitrite. It is now found that k_8/k_4 for lactoperoxidase is approximately 9, confirming that compound II is the rate-limiting intermediate when pyrogallol is the donor.

Different forms of lactoperoxidase

After the present work was completed, a paper by Polis & Shmukler (1953) described the purification of the enzyme largely by chromatographic methods. Examination of their preparation by electrophoresis and by spectrophotometry revealed the presence of two forms of lactoperoxidase distinguished by their different mobility in acetate buffer, by their absorption ratios at 412 and 280 mμ. and by their different turnover numbers in the reaction with hydrogen peroxide and dihydroxyphenylalanine. In the present study it was found that when the enzyme was approximately 40–50 % pure, further purification inevitably led to inactivation of the enzyme. The value of the comparison of specific activity with the spectrophotometrically determined enzyme-haem concentration is thus emphasized (see Table 2).

SUMMARY

1. A spectrophotometric method of measuring the activity of lactoperoxidase using pyrogallol as hydrogen donor is described. The conditions of measurement are such that, with high donor concentration and low peroxide concentration, the reaction of enzyme with peroxide is measured.

2. A brief description is given of the preparation of lactoperoxidase of high spectroscopic purity in the visible region of the spectrum and of 40–50 % absolute purity.

3. The absorption curves in the visible and Soret regions are given for lactoperoxidase and its carbon monoxide-, cyanide-, fluoride-, and pyridine-haemochromogen derivatives.

4. The absorption spectra are very similar in pattern to those for the protohaematin compounds, horse-radish peroxidase, catalase and methaemoglobin. The prosthetic group of lactoperoxidase is probably a haematin closely related to protohaematin. The absorption bands of lactoperoxidase and of its derivatives lie further to the red than the protohaematin enzymes indicating that the prosthetic group is not identical with protohaematin.

5. The kinetics of lactoperoxidase have been examined mathematically using the mechanism of peroxidase action described by Chance and modified by George.

6. The Michaelis constant ($1{\cdot}08 \times 10^{-3}$ M) was determined experimentally and found to be in good agreement with the value calculated by Chance.

I wish to thank Prof. D. Keilin, F.R.S., for his interest in this work and Dr E. C. Slater and Dr E. F. Hartree for much helpful advice. I also wish to thank the Australian National University for a scholarship.

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The Isolation and Estimation of the Steroid Oestrogens in Placental Tissue

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Work on the biosynthesis of the natural oestrogens has been rendered very difficult in the past by the lack of quantitative methods of purification of the microgram quantities of these hormones which exist in tissue. Though there were several methods of estimating the pure oestrogens, they were not directly applicable to accurate estimations in urinary or tissue extracts because of the presence of impurities.

The rigorous purification of oestrone, oestradiol and oestriol has been the aim of several recent investigations (Engel, Slaunwhite, Carter & Nathanson, 1950; Boscott, 1951; Swyer & Braunsberg, 1951; Bauld, 1952) all of which deal with urinary extracts, whilst Diczfalusy (1953) and Ryan & Engel (1953*a*, *b*) have recently applied the countercurrent distribution method of Engel *et al.* (1950) to tissue extracts. In this technique, however, nonspecific fluorogenic material present, particularly in the early fractions, makes difficult the estimation of minute quantities of oestrogen by fluorimetry.

The methods to be described were developed in order to make possible further work on the *in vitro* synthesis of oestrogens in tissue slices. Initially this had been investigated by using a bio-assay. The normal method of injecting the oestrogen into spayed, adult female mice had not proved to be sufficiently sensitive, but the intravaginal insertion technique of Emmens (1941) produced 50% vaginal cornification with 1·0 μmg. oestrone, and by this means it had been shown that full-term human placental tissue contained 30 Emmens mouse units/g. When this placental tissue was incubated in Warburg flasks for 3 hr., the oestrogenic potency increased by approximately 100%. However, the large number of mice required for assay, and the lack of specificity, made it clear that chemical estimations were required.

After a study of existing chromatographic techniques, a method was developed which involved a preliminary chemical separation followed by paper partition chromatography and a special spotting technique, with elution of the spots and estimation by fluorimetry.

A preliminary account of part of this work has already been published (Mitchell, 1952).

EXPERIMENTAL AND RESULTS

Reagents

Ether (May and Baker Ltd., Dagenham), A.R., peroxide-free, was refractionated through a 100 cm. column packed with single-turn glass helices, and used the same day. Benzene, A.R., thiophen-free, was refluxed for 30 min. with conc. H_2SO_4 and refractionated through a similar 60 cm. column. This column was also used to refractionate the AnalaR carbon tetrachloride and methanol. Ethanol was B.P. quality (James Burrough Ltd., London). Diazotized *p*-nitrobenzeneazodimethoxyaniline (Fast Black Salt K) was from the Clayton Aniline Co. Ltd., Manchester. All other reagents were of AnalaR quality.

Extraction procedure

The procedure developed in the course of this work is described in Fig. 1. Fresh placenta was minced and mixed with an equal volume of 80% (v/v) ethanol within 30 min. of delivery. The method of extraction separated the oestrogens into 'free' (1), 'conjugated' (2), and 'protein-bound, (3) fractions (Diczfalusy, 1953). These were each further

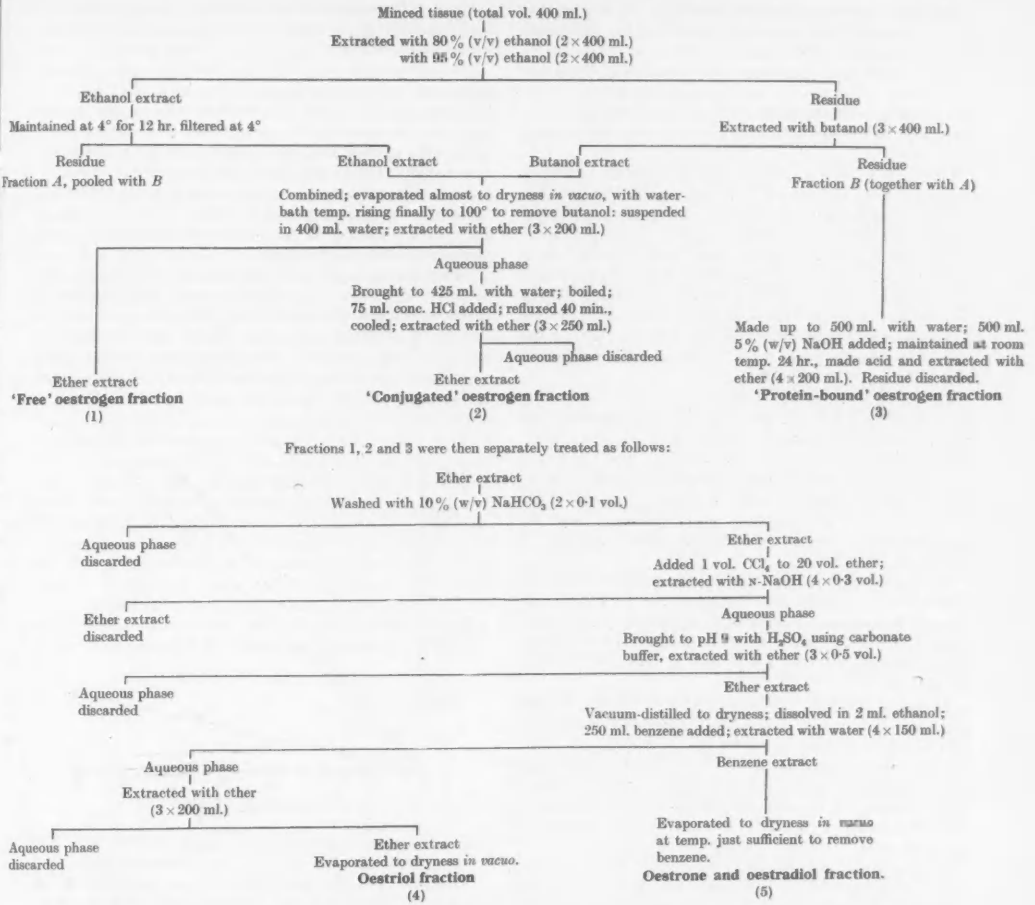
Minced tissue (total vol. 400 ml.)
Extracted with 80% (v/v) ethanol (2×400 ml.) with 95% (v/v) ethanol (2×400 ml.)
Ethanol extract
Maintained at 4° for 12 hr. filtered at 4°
Residue
Extracted with butanol (3×400 ml.)
Residue
Fraction A, pooled with B
Ethanol extract
Butanol extract
Residue
Fraction B (together with A)
Combined; evaporated almost to dryness in vacuo, with water-bath temp. rising finally to 100° to remove butanol: suspended in 400 ml. water; extracted with ether (3×200 ml.)
Aqueous phase
Brought to 425 ml. with water; boiled; 75 ml. conc. HCl added; refluxed 40 min., cooled; extracted with ether (3×250 ml.)
Aqueous phase discarded
Made up to 500 ml. with water; 500 ml. 5% (w/v) NaOH added; maintained at room temp. 24 hr., made acid and extracted with ether (4×200 ml.). Residue discarded.
Ether extract
'Free' oestrogen fraction
(1)
Ether extract
'Conjugated' oestrogen fraction
(2)
'Protein-bound' oestrogen fraction
(3)
Fractions 1, 2 and 3 were then separately treated as follows:
Ether extract
Washed with 10% (w/v) NaHCO3 (2×0·1 vol.)
Aqueous phase discarded
Ether extract
Added 1 vol. CCl4 to 20 vol. ether; extracted with N-NaOH (4×0·3 vol.)
Ether extract discarded
Aqueous phase
Brought to pH 9 with H2SO4 using carbonate buffer, extracted with ether (3×0·5 vol.)
Aqueous phase discarded
Ether extract
Vacuum-distilled to dryness; dissolved in 2 ml. ethanol; 250 ml. benzene added; extracted with water (4×150 ml.)
Aqueous phase
Extracted with ether (3×200 ml.)
Benzene extract
Aqueous phase discarded
Ether extract
Evaporated to dryness in vacuo.
Oestriol fraction
(4)
Evaporated to dryness in vacuo at temp. just sufficient to remove benzene.
Oestrone and oestradiol fraction.
(5)

Fig. 1. Extraction of 'free', 'conjugated' and 'protein-bound' oestrogens from tissue, and their purification and separation into oestriol, and oestrone:oestradiol fractions. All extracts were stored at $-20°$ when not being actually handled. All processes were carried out at room temp. except where stated. Before distillation of solvents, extracts were first evacuated at room temp. to remove air, and the distillation subsequently carried out in a water-pump vacuum with a fine stream of nitrogen bubbles.

separated into an oestriol (4) and an oestrone plus oestradiol (5) fraction. These fractions (4 and 5) were each dissolved in 5 ml. of 90% (v/v) methanol and washed with 5 ml. of pentane; the pentane was back-extracted three times with 5 ml. 90% (v/v) methanol and the pooled methanol evaporated to dryness at 70° in a stream of N_2. The residue was taken up in 0·2 ml. of ethanol for chromatography. The pentane wash (Ryan & Engel, 1953*a*) is not necessary for the 'protein-bound' fraction. The reason for its inclusion is to remove remaining greasy materials, which if present in large amounts (more than 5 mg.) in the final extract cause spreading of the spot on the chromatogram during equilibration.

The original ethanol extracts from 400 g. placental tissue contained approx. 16 g. of material, which was finally reduced to 2–10 mg. for chromatography.

Initial investigation of techniques for further purification and estimation

Coupling with azo dye. Heftmann (1950) separated pure oestrogens by coupling with a saturated aqueous solution of Fast Black Salt K to make azo compounds, followed by paper chromatography. Our sample of this salt solution had to be diluted 30 times with water to achieve results similar to those of Heftmann (1950).

In order to investigate the possibility of eluting the purple spots from the paper and estimating the oestrogens colorimetrically, an effort was first made to increase the R_F value of oestriol and decrease that of oestrone. These are respectively 0·07 and 0·95, and the spots tended in the one case to merge with the residue left on the origin, and in the other with an orange band which moved with the moving-phase front.

The oestrogens were first separated into an oestrone: oestradiol and an oestriol fraction by the preliminary extraction procedure. Heftmann's (1950) original solvent mixture was 200 ml. of toluene, 100 ml. of light petroleum (b.p. 35–60°), 30 ml. of ethanol and 70 ml. of water; after equilibration the top layer was used for the moving phase. This system was modified by increasing the amount of light petroleum to 250 ml. for the oestrone and oestradiol mixture, and decreasing it to 50 ml. for oestriol. The R_F values in the respective systems were then: oestrone, 0·91; oestradiol-17β, 0·63; and oestriol, 0·30.

The parts of the paper containing the spots were cut up and placed in 5 × 0·5 in. test tubes, 1 ml. of benzene was added, and the tubes were gently shaken for a few min.; 0·5 ml. of the benzene was then removed and the colour density measured in the 0·5 ml. cells of the Spekker Photoelectric Absorptiometer using an Ilford Spectrum Blue-green 603 filter (transmission max. 490 mμ.). The absorptions were measured against extractions from controls containing no oestrogen.

Fig. 2 shows that this method may be used for the separate estimation of oestrone and oestradiol, but for oestriol the slope of the calibration curve was very small, and efforts to make the reaction more sensitive failed. More accurate measurements could be made with a Beckman Spectrophotometer.

Attempts to couple the oestrogens present in the oestrone and oestradiol fraction of placental extract with Fast Black Salt K and to chromatograph the products gave long, dark-brown streaks on the paper. With the oestriol fraction, a spot was formed corresponding in every respect to that of pure oestriol, but the amount present could not be determined precisely by the above method because the slope of the calibration curve was so small (see Fig. 2).

Pure oestrone and oestradiol were added to the fraction which contained these oestrogens, and the mixture was coupled with Fast Black Salt K. The product still did not produce any spots on the chromatogram, and this shows that something present in the extract interfered with the method.

Countercurrent distribution. This method of Engel *et al.* (1950) for separating oestrogens in urinary extracts proved unsuitable for the analysis of normal placental extracts because of a large, but variable, non-specific background fluorescence. Diczfalusy (1953) has used this method, but points out that some overestimation occurs at low concentration of oestrogen, and that oestrone cannot be quantitatively separated from the non-specific fluorogenic contaminants.

Chromatography on alumina-impregnated paper. Using this technique (Bush, 1950), a method of ascending two-dimensional chromatography was evolved which would separate, on papers 6 in. square, part of the oestrogens reasonably free from impurities. The solvents were run to the edge of the paper and the separation achieved is shown in Fig. 3. The position of the oestrogens was found by the iodine method (Bush, 1950).

Many of the original disadvantages of this method have been removed (Bush, 1952), but small amounts of impurity still cause alteration of R_F values with streaking.

Chromatography by Celite:sodium hydroxide column. Apparently reliable results have been obtained by Swyer & Braunsberg (1951) when this method was applied to the very low amounts of oestrogens found in non-pregnancy urines. The method has been further perfected for urine by Bauld (1952, 1953), and Bitman & Sykes (1953) have investigated its use for separating pure oestrogens. When the technique was applied to tissue extracts, large amounts of non-specific, fluorogenic material still remained in the oestrogen-containing fractions.

Chromatography on paper impregnated with glycerol and propylene glycol and on a rubber column. Investigation of these techniques (Boscott, 1951; Nyc, Maron, Garst & Friedgood, 1951) showed that, though good separations were produced using pure oestrogens, tissue extracts gave considerable spreading and tailing of the spots in the paper method, and in both cases there was insufficient removal of impurities.

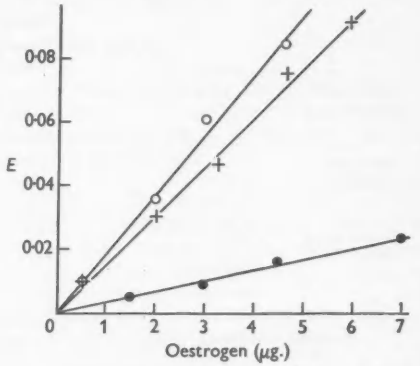

Fig. 2. Standard curves for oestrogens coupled with Fast Black Salt K after elution from chromatograms with benzene. ○—○, oestradiol; +—+, oestrone; ●—●, oestriol. Oestradiol and oestrone measured in 0·5 ml. cups of a Spekker Photoelectric Absorptiometer; oestriol in a Beckman Spectrophotometer. The absorptions were measured against extractions from blank runs containing no oestrogen.

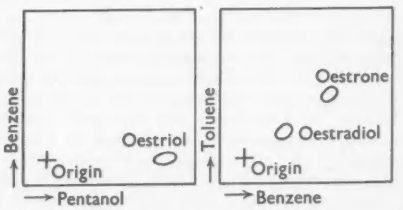

Fig. 3. Two-dimensional chromatography of oestrogens on alumina paper (after Bush, 1950). For further details see text. The spots were detected by iodine and drawn in before they faded.

Reversed-phase partition chromatography on silicone-treated paper. This method (Kritchevsky & Tiselius, 1951), gave perfect results for pure steroids, but impurities caused streaking and drastic alteration of R_F values.

Reduction of extracts by lithium aluminium hydride. Some of the impurities in the 'phenolic' fractions may have been weak acids containing carboxyl groups. If these acidic groups could be reduced by means of lithium aluminium hydride, then these impurities could be easily removed.

Much impurity was removed from the extract by this means, but further purification was still necessary as shown by the amount of residue, and by the results of coupling with Fast Black Salt K. In the reduction process, oestrone was quantitatively reduced to oestradiol as judged by the intensity of the spot after coupling.

The use of Folin & Ciocalteu's reagent. If the oestrogens were heated with this phenol reagent (Folin & Ciocalteu, 1927), the colour changed from yellow to green, and became blue on addition of alkali (see Fig. 4).

For quantitative assay, the oestrogen was dissolved in ethanol, placed in 5 × 0·5 in. test tubes, the ethanol removed in a current of nitrogen and 0·4 ml. of the reagent (diluted with 2 vol. water) added. The tubes were then stoppered with cotton wool, heated in a boiling-water bath for 10 min., cooled and transferred to a water bath at 25°; 0·15 ml. of 25% (w/v) Na_2CO_3 solution was added, the stoppers were replaced, and the tubes left for 10 min. The colour intensity was then measured within 5 min. in the 0·5 ml. cells of the Spekker instrument, using an Ilford Spectrum Red 608 filter (transmission max. 680–700 mμ.) (Fig. 4). Controls must always be measured. All three oestrogens give similar extinction values. When a fluorimeter was not available (see later), this method proved reliable for the estimation of pure oestrogens. However, its low degree of specificity, and susceptibility to small quantities of foreign matter, renders it inferior to fluorimetry for general use with tissue extracts.

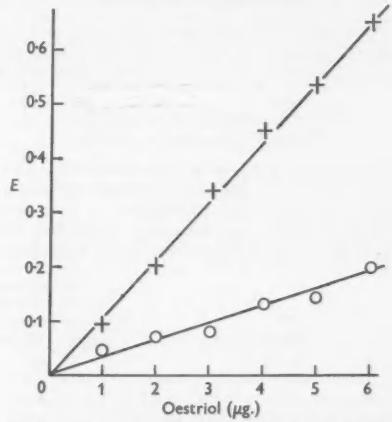

Fig. 4. Standard curves for oestriol treated with Folin & Ciocalteu's reagent. To oestriol, 0·4 ml. of diluted Folin & Ciocalteu's reagent was added. Tubes were heated in boiling-water bath for 10 min. ○—○ (green colour). To this solution was added 0·15 ml. 25% Na_2CO_3 and the tubes were kept in bath at 25° for 10 min. +—+ (blue colour). All absorptions were measured against blank solutions containing no oestrogen, and all readings were taken in region of max. absorption (690 mμ.) using 0·5 ml. cells in a Spekker Photoelectric Absorptiometer (filter Ilford 608, transmission max., 680–700 mμ.).

Purification by paper chromatography

General procedure; apparatus and methods. The following method of paper chromatography was found to give a quick and simple separation, and almost complete purification of the three oestrogens in the tissue extracts. To avoid streaking, and to give reproducible R_F values in the presence of the large amount of greasy impurity found in the extracts, it was found essential to use an elevated temperature under the carefully controlled conditions recently described by Bush (1952) for adrenal steroids. All chromatography tanks were enclosed in a cabinet (dimensions 100 × 70 × 70 cm. high), controlled thermostatically at 32 ± 0·5° (Bush, 1952). Good results have also been obtained with a well-insulated tank placed in a laboratory incubator. The dimensions of the glass tanks were 30 × 20 × 40 cm. high, and to ensure proper equilibration of the highly volatile solvents at the relatively high temperature used, the inner walls of the tank were covered with wads of filter paper. Those at the sides dipped into the mobile phase covering the bottom, while those at the ends dipped into vessels containing 200 ml. of stationary phase (cf. Bush, 1952).

Spreading of the spots due to evaporation of solvent through the lid seal was reduced by covering the tank lid with a piece of cloth, the edges of which hung a few inches down the outside of the tank.

The chromatograms were hung with approx. 6 cm. of paper running with a slope of 1 in 1 between the liquid level and the supporting rod. The starting line was 2 cm. beyond this rod (cf. Block, LeStrange & Zweig, 1952).

Spotting technique. The final extracts in ethanol (Fig. 1), were placed on strips 5 × 18 in. of Whatman no. 541 filter paper, and the ethanol was evaporated by a stream of air directed under the paper. For quantitative work, the paper must previously be extracted with methanol in a Soxhlet apparatus for 48 hr. to reduce the amount of impurities soluble in methanol.

Up to 20 μg. of each oestrogen could be run on the paper before any sign of streaking appeared; the minimum amount for detection is 0·5–1·0 μg. Single preparative runs of extracts from 100 g. of placental tissue have been made, but, for estimations, extracts from 10–20 g. were placed on the paper.

Solvent systems. The mobile phase for oestrone and oestradiol was made from 1000 ml. light petroleum (b.p. 100–120°); the stationary phase from methanol (1000 ml.). For oestriol these were respectively from benzene (1000 ml.) and from a mixture of methanol (500 ml.) and water (500 ml.). The phases were mixed, equilibrated overnight in the cabinet, separated, and placed in their respective compartments in the tanks (except in the running troughs). A store of the equilibrated mixture was kept in the cabinet to replenish the tanks from time to time.

Equilibration of paper. If the tank were opened during insertion of the paper for the minimum time only, not more than 5 min. were needed for equilibration.

Running of the chromatograms. The runs were started by pouring 35 ml. of mobile phase into the troughs by means of a funnel through holes in the lid of the tank (Hanes & Isherwood, 1949). The solvent systems have been so

arranged that the runs may take place overnight. With a 15 hr. run, the spots were well distributed over the papers. The R_F values in the respective systems are oestrone, 0·2; oestradiol-17β, 0·07; and oestriol, 0·13 (Fig. 5).

Detection of spots

Methods already described. The detection of oestrogens on paper chromatograms has always proved difficult. The iodine technique described by Bush (1950) will detect 1 μg. of steroid/cm.^{2}. The method is however by no means specific, and the spots disappear after a few hours owing to evaporation of the iodine. Boscott (1951) either immersed the paper in, or spotted it with, 90% (v/v) H_2SO_4, and looked for fluorescence under ultraviolet light.

Detection by ultraviolet absorption. A 125 w mercury-vapour lamp with filters, Chance OX7; and Cl_2, 1·25 M-$NiSO_4$ and 0·35 M-$CoSO_4$, each contained in 4 cm. diam. quartz flasks, gives sufficient light of the right wavelength (approx. 281 mμ.) to detect 2 μg. of oestrogen/cm.^{2}. The exposure of 30 sec. is made on Kodak Reflex Contact Document Paper placed behind the chromatogram at a distance of 1 m. from the lamp. The specificity is poor, but this method can be used for relatively large amounts of oestrogen, and has the advantage that the oestrogen is left intact for subsequent assay.

Detection by the use of Folin & Ciocalteu's reagent. This reagent gives a sensitive and satisfactory method for the detection of oestrogens on paper chromatograms. It is not necessary to apply heat, and, for the alkali, vapour from ammonia solution is preferable to a spray of Na_2CO_3, as the latter tends to produce a darker background and spoils the texture of the paper.

The paper was lightly sprayed with Folin & Ciocalteu's reagent (freshly diluted, 1 vol. reagent to 5 vol. water), and placed immediately into a glass tank, the bottom of which was covered with NH_3 soln. (sp.gr. 0·880). When the spots reached maximum intensity (about 5 min.), the paper was removed and dried at room temp. to give a permanently stable chromatogram; the oestrogens showed as blue spots on a pale-blue background (Fig. 5). The minimum amount detectable is 0·5 μg./cm.^{2}. The spray used for applying the reagent must be all of glass or Bakelite construction, as many metals react with the reagent to give a dark-blue background to the chromatogram.

Though the blue colour is formed by many other substances besides phenols, we found that, within the group of steroids investigated, in addition to the oestrogens, only those bearing an α-ketol side chain gave a strong colour (Table 1).

For quantities of oestrogen over 1 μg. the reaction does not go to completion during a single spraying, consequently, for amounts more than this, the depth of colour on the paper is only quantitative if the paper is put through the complete process (drying after each treatment with NH_3) at least 4 times. The oestrogen content of the spots can then be obtained by visual comparison with standards run in parallel. Alternatively, the amount of oestrogen present may be estimated after the first spraying by cutting the spot into small pieces, adding reagent and treating as previously described under the estimation using Folin & Ciocalteu's reagent.

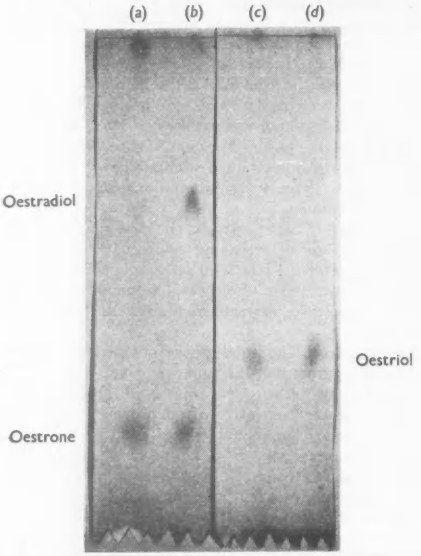

Fig. 5. Chromatograms of placental extracts developed in benzene, light petroleum, methanol and water systems: (*a*) 'free' oestrone and oestradiol from 50 g. placental tissue; (*b*) 5 μg. pure oestrone and oestradiol; (*c*) 'free'-oestriol fraction from 50 g. placenta; (*d*) 5 μg. pure oestriol. Spots made visible with Folin and Ciocalteu's reagent.

Fluorimetric estimation

For this method the positions of the spots were found by means of parallel runs with smaller amounts of similar extract, and of pure oestrogens. The strips containing these

Table 1. *Specificity of steroids to Folin & Ciocalteu's reagent*

The steroid was placed on Whatman 541 filter paper in ethanolic solution, the ethanol was allowed to evaporate, the paper was lightly sprayed with Folin & Ciocalteu's reagent diluted with 5 vol. water and immediately placed for 5 min. in the vapour from NH_3 solution, sp.gr. 0·880.

Steroid	Min. amount detectable by spray technique (μg./cm.^{2} of paper)
Oestrone	0·5
Oestriol	0·5
Oestradiol-17β	0·5
17α-Hydroxy-11-dehydrocorticosterone (cortisone)	2·0
Deoxycorticosterone	5
17α:21-Dihydroxypregnane-3:11:20-trione 21-acetate (4:5-dihydrocortisone acetate)	20
17α-Hydroxy-11-dehydrocorticosterone 21-acetate (cortisone acetate)	50
Dehydro*epi*androsterone	50
Androsterone	100
Progesterone	100
3β:21-Dihydroxypregn-5-en-20-one 21-acetate (21-acetoxypregnenolone)	100
Pregnane-3α:20α-diol	100

parallel runs were cut out and sprayed with Folin & Ciocalteu's reagent. The smallest area of paper ensuring removal of all oestrogen in the main runs was then cut out, together with similar areas from a blank part of the chromatogram. Standard amounts of oestrogen were placed on these blank papers.

Despite pre-extraction with methanol for 48 hr., it was found necessary to extract each paper with the same amount of solvent for a standard time, and for this purpose an apparatus was constructed which dripped 5 ml. of methanol from capillary tubes over a period of 8 min. The drops were allowed to fall on the top corners of the papers, each suspended on a stainless-steel hook, and collected from the bottom corners into the test tubes in which the fluorescence was to be developed. The methanol was removed by a stream of N_2 with the tubes immersed in a water bath at 70°.

Several methods for the fluorimetric determination of the oestrogens have been described (Finkelstein, Hestrin & Koch, 1947; Jailer, 1948; Bates & Cohen, 1950; Engel *et al.* 1950; Diczfalusy, 1953). Each of these techniques was investigated, and that of Diczfalusy (1953) found to be the most satisfactory for general use. In this method the oestrogen is dissolved in 0·5 ml. ethanol and the fluorescence developed by heating for 10 min. in a boiling-water bath with 8 ml. of 88% (v/v) H_2SO_4 followed by cooling in cracked ice.

A sensitive fluorimeter, suitable for estimating the fluorescence produced by amounts down to 0·1 μg. oestrogen, which has given satisfaction during 3 yr. general use, was constructed from readily available materials utilizing the photomultiplier circuit described by Lowry (1947). With Chance O.N. 20 (heat) and Chance O.B. 10 (transmission max. 425 mμ.) as primary filters, and Ilford Spectrum Green 604 (transmission max. 520 mμ.) as secondary filter, the sensitivity of the instrument was such that a solution of 0·05 μg. sodium fluoresceinate/ml. gave a current of 0·5 μA and half-scale deflexion (8 cm.) on the galvanometer.

The development of the fluorescence is very susceptible to external influences, but the work of Braunsberg (1952), Brown (1952) and of Diczfalusy (1953), has shown that if these influences are controlled, good results can be obtained. This control entails completing a standard graph with each set of estimations, because we have found, in agreement with Braunsberg (personal communication), that graphs produced at different times may vary considerably.

Final identification of oestrogens from placentae

The extracts from 160 g. of placental tissue ('free' fraction Fig. 1) were run on chromatograms. The spots were removed from the paper and eluted as above. The methanol was evaporated, the residue dissolved in 1 ml. of ethanol and the ultraviolet absorption spectra were measured and compared with those for pure oestrogens (Fig. 6).

It was found that the filter paper, even after previous extraction in the Soxhlet extractor for several days, still contained a substance which was extracted by methanol and absorbed strongly in the ultraviolet region, causing distortion of the oestrogen curve. Extracts were therefore made of similar blank areas of filter paper and used as the solvent blank for obtaining the absorption curves.

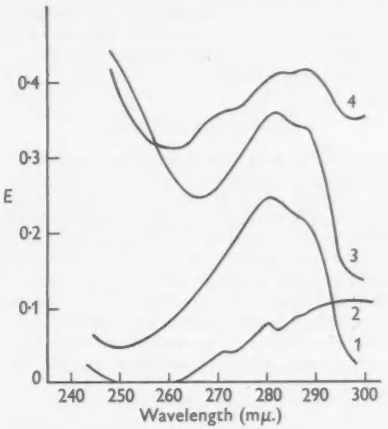

Fig. 6. Absorption spectra of oestrogen spots eluted from chromatograms and compared with pure oestrone. 1, 30 μg. pure oestrone/ml. ethanol; 2, oestradiol; 3, oestriol and 4, oestrone from 160 g. placental tissue, dissolved in 1 ml. ethanol. The absorptions were measured against extractions from similar areas of blank chromatogram paper. The absorption curves for pure oestriol and oestradiol are similar to that for oestrone. Readings were taken at every mμ. over the whole range shown and the curves are drawn through the experimental points.

Table 2. *Comparison of oestrogen in paper-chromatogram spots from* 400 *g. placental tissue when assayed by various methods*

Oestrogens were extracted and purified by separation in solvents and the final products were chromatographed in benzene, light petroleum, methanol and water systems as described in the text.

Method of assay	Oestriol (μg./kg.)	Oestrone (μg./kg.)	Oestradiol (μg./kg.)	Estimated error of method (%)
Visual comparison of spots obtained with Folin & Ciocalteu's reagent	40	16	4	±10
Absorption in ultraviolet (with allowance for estimated impurity)	40	25	4	±20
Biological (cornification of mouse vagina)	25–60*	17	1–4*	±10
Fluorimetry	40·5	13·8	5·0	± 5

* The amounts of material available for assay were insufficient for more accurate estimations.

Although peaks were found for oestriol and oestrone, the oestradiol content of the placental extracts was always low, and in this case was insufficient to produce a clear peak at 281 mμ.

The ethanol was then removed from these solutions, and the residue coupled with Fast Black Salt K, followed by chromatography (Heftmann, 1950). In each case well-defined spots were produced, identical in all ways with spots produced by parallel runs of the three pure oestrogens.

The absorption curves were obtained for similar eluates which were then assayed biologically (by vaginal cornification produced by injection into adult, spayed female mice) and fluorimetrically, with the results shown in Table 2.

Thus, in addition to the spots having the same R_F values as pure oestriol, oestrone and oestradiol, their identity has been proved by ultraviolet absorption, chromatography of the product obtained by coupling with azo dye, development of fluorescence in H_2SO_4 and by bio-assay, and good agreement of results has been obtained by the various methods of estimation (Table 2).

Recovery experiments

As preliminary work showed there was a considerable loss of the oestrogens during purification, the recovery of the three oestrogens added to full-term placental tissue was investigated. The importance of the rigorous purification of all solvents, the necessity of completing all processes above room temperature under N_2, and the need to keep extracts in the deep freeze (temp. $-20°$) whenever possible was proved early in the investigation. These precautions, however, did not prevent a large loss, and to arrive at the true level of oestrogen in the samples of placental tissue, it was necessary to complete several large-scale recovery experiments (Table 3).

The oestrogens were added in ethanolic solution after the first addition of 80% (v/v) ethanol to the tissue (see Fig. 1), and, as Diczfalusy (1953) has also shown, the added oestrogen did not appear in the 'conjugated' or 'protein-bound' fractions. Heating the tissue to 100° in N_2 before adding the oestrogen increased the recovery in all cases, whilst heating in air increased it further, but destroyed some of the original oestrogen. The use of thiourea did not improve the recovery, though Bauld (1953) found that some antioxidants prevent losses at certain stages in the extraction of oestrogens from urine.

Though recoveries varied from batch to batch, the good agreement of duplicate estimations carried out on the same specimen (Table 4), shows that the losses involved in purification were constant for each batch of minced placenta.

Very little loss took place during chromatography and elution from the chromatogram. Oestrogens added to the extract placed on the paper were recovered as follows: oestriol, 97%; oestrone, 95%; oestradiol, 84%.

The experimentally determined values of free oestrogen per kg. in seven full-term placentae were: oestriol, 190 μg.; oestrone, 86 μg.; oestradiol, 25 μg. When these values are corrected for the very low recoveries found in the appropriate experiments (see Table 4), the estimated values are: oestriol, 680 μg.; oestrone, 580 μg.; oestradiol, 170 μg. Since these estimated values are based on very low recoveries, and since those recoveries vary widely from one sample to another (for example, between 18·5 and 49·4% for oestriol, and between 4·5 and 20% for oestrone), the authors wish to emphasize that the recovery figures may only be very roughly applicable to other samples of placental tissue.

The corrected amounts lie between 4 and 16 times more than was reported by Huffman, Thayer & Doisy (1940), working with more than 400 kg. of placental tissue. They found respectively 140, 35 and 38 μg./kg. of each oestrogen. Diczfalusy (1953) found the amounts of the free oestrogens to be respectively 125·4, 46·7 and 3·1 μg./kg. Neither of these workers corrected for losses during purification.

As recovery experiments were not carried out on the 'conjugated' and 'protein-bound' fractions, losses in purification cannot be applied to the average results for these fractions shown in Table 4.

To ascertain how much of the oestrogen in the placental tissue was due to the small amount of retained blood, estimations were carried out on 10 ml. samples of venous blood taken just before or after delivery. The sensitivity of the method was not sufficient to give accurate results on such small amounts of material, but was sufficient to show

Table 3. *The recovery of 'free' oestriol, oestrone and oestradiol added to full-term human placental tissue*

The pure oestrogen was added to the mince in ethanolic solution immediately after the addition of 80% (v/v) ethanol for extraction.

			Recovery of added 'free' oestrogen					
			Oestriol		Oestrone		Oestradiol	
Expt. no.	No. of placentae pooled	Treatment of placentae before extraction	Amount added (μg.)	Amount recovered (%)	Amount added (μg.)	Amount recovered (%)	Amount added (μg.)	Amount recovered (%)
1	3	None	500*	22·6	500*	18·0	500*	11·4
		None	30*	19·4	30*	16·0	30*	12·0
		Heated 25 min. at 100° in N_2	500*	37·7	500*	37·2	500*	20·4
			30*	68·0	30*	32·6	30*	18·0
2	2	None	500†	49·4	500†	20·0	500†	13·5
		None	30†	37·3	30†	14·3	30†	17·3
5	2	None	20*	18·5	20*	4·5	20*	15·0
		Heated 25 min. at 100° in air	20*	99·0	20*	13·0	20*	70·0
Average recovery from untreated placentae				26·7		13·5		13·7

* Added to 150 g. placental tissue. † Added to 195 g. placental tissue.

Table 4. *Experimental values for 'free', 'conjugated' and 'protein-bound' oestriol, oestrone and oestradiol, with values corrected by recovery experiments (cf. Table 3) for the 'free' fraction with the same placentae*

For details see text.

Expt. no.	No. of placentae pooled	Treatment of placentae before extraction	'Free' oestrogen isolated (μg./kg.)			'Conjugated' oestrogen isolated (μg./kg.)			'Protein-bound' oestrogen isolated (μg./kg.)			'Free' oestrogen content corrected on basis of recovery expt. (μg./kg.)		
			Oestriol	Oestrone	Oestradiol	Oestriol	Oestrone	Oestradiol	Oestriol	Oestrone	Oestradiol	Oestriol	Oestrone	Oestradiol
1	3	None	157	108	0	65	25	0	33	16	0	748	635	0
		Heated 25 min. at 100° in N_2	168	111	0	68	23	0	31	16	0	317	317	0
2	2	None	205	112	14	—	—	—	—	—	—	416	560	106
		None	234	106	12	—	—	—	—	—	—	627	741	72
3	1	None	300	109	24	61	33	13	14	27	23	—	—	—
		None	300	100	24	79	34	14	39	27	24	—	—	—
4	2	Heated 25 min. at 100° in N_2 + thiourea to 0·1%	288	194	10	—	—	—	—	—	—	—	—	—
		Heated 25 min. at 100° in N_2	275	194	8	—	—	—	—	—	—	—	—	—
5	2	None	148	20	76	0	8	17	0	5	10	730	440	507
		Heated 25 min. at 100° in air	92	19	57	0	8	8	0	1	17	93	146	82
Average content of untreated placentae			190	86	25	44	21	8	21	14	7	678	584	170

that the maternal blood contains considerably less free oestrogen (10–50 μg. of each oestrogen/kg.) than was found in placentae. This is in agreement with the values reported by Diczfalusy (1953) and Veldhuis (1953).

The following procedure was developed to find the approximate amounts of maternal and foetal blood in placental tissue. The maternal blood in the sinuses was obtained by washing the placenta (approx. 300 g.) 10 times, with gentle massage, in 30 ml. quantities of water. The placenta was then finely minced and extracted 10 times with 50 ml. quantities of water. Haemoglobin was measured in the washings by the method of Haldane (1901). Haemoglobin estimations were also carried out by the method of Clegg & King (1942) on the mother and baby soon after delivery. The results show that the tissue contained approximately 6% foetal, and 3% maternal blood. Assuming that the foetal-blood oestrogen is not higher than the level in maternal blood, the blood content of the placental tissue could not account for more than 5 μg./kg. of each oestrogen. This (uncorrected) content is small compared with the amounts found in placenta.

DISCUSSION

By the use of paper chromatography, two of the great technical difficulties involved in the chemical estimation of the minute quantities of oestrogens in tissue have been largely solved; namely the complete separation of the three oestrogens, and their preparation in a sufficient state of purity. This is shown by the concordant results obtained by the four widely different methods of estimation given in Table 2.

Some of the possible errors and losses at various stages of purification have been extensively discussed by Diczfalusy (1953). It is difficult to see how these can be further eliminated except perhaps by the use of suitable antioxidants, or of labelled oestrogens for use as internal standards to measure the losses. However these are not yet readily available.

The corrected oestrogen contents of full-term placentae present quite a different picture from that shown by the previously reported uncorrected values (Huffman *et al.* 1940; Diczfalusy, 1953) which suggested that there was more than 3 times as much oestriol as oestrone or oestradiol. The corrected figures show that oestrone is present in similar amount to oestriol, and that there is much more oestradiol than previously suspected.

Recently, Ryan & Engel (1953*b*) have shown that sliced, full-term placental tissue, when incubated separately with relatively large amounts of each of the three oestrogens, converts oestradiol into oestrone and degrades both to unrecognizable metabolites. Oestriol was recovered almost quantitatively after incubation. It is possible that some conversion and destruction is taking place during the interval between delivery and extraction of the tissue, so the original level of oestradiol may be higher when the placenta is *in situ*.

The advantages of the method described in this paper, and also that of Diczfalusy (1953), are that they require very much smaller amounts of material and take far less time than the earlier procedures (cf. Huffman *et al.* 1940). The method is being applied to the separation and estimation of oestrogens in urine.

SUMMARY

1. An investigation has been made into many of the chromatographic techniques now available, with a view to their use in the separation and purification of tissue oestriol, oestrone and oestradiol.

2. A method has been developed for the isolation and estimation of these three oestrogens in placental tissue. It involves a preliminary chemical separation followed by paper chromatography, elution and fluorimetric estimation.

3. The use of Folin & Ciocalteu's reagent in a sensitive method for detection of the oestrogens on paper chromatograms is described.

4. The use of azo-dye coupling followed by paper chromatography (Heftmann, 1950), has been adapted to the quantitative assay of oestrone and oestradiol, and a new method of assay for oestriol, oestrone and oestradiol using Folin & Ciocalteu's reagent is described.

5. The uncorrected average quantities of 'free' oestrogen/kg. in seven full-term placentae were: oestriol, 190 μg., oestrone, 80 μg. and oestradiol, 25 μg. The values for 'conjugated' oestrogen were respectively: 44, 21 and 8 μg., and for 'protein-bound' oestrogen: 21, 14 and 7 μg. Large differences were found in the oestrogen content of individual placentae.

6. Recovery experiments show that the normal methods of extraction and purification, when applied to the 'free' oestrogens in full-term placental tissue give losses of between 70 and 90%. The corrected average quantities of 'free' oestrogen/kg. in seven full-term placentae were estimated as: oestriol, 680 μg., oestrone, 580 μg. and oestradiol, 170 μg./kg. Whilst these corrections are accurate for the seven placentae examined, they may be only roughly applicable to other samples of this tissue.

We wish to thank Prof. H. A. Krebs, F.R.S., and Dr C. G. Paine for their interest and encouragement in this work, also Mr R. J. Hodgins for expert technical assistance, and Mr D. E. Hughes, Mr D. Eyre and Mr A. Renshaw for help in the construction of the fluorimeter. We are also grateful to Dr J. K. Norymberski (Sheffield), Dr Hoffman, Ciba Ltd. (Basle), Dr D. McGinty, Parke Davis, Ltd. (Detroit), and Dr W. J. Tindall, Organon Ltd. (London), for gifts of steroids, to the Clayton Aniline Co. (Manchester), for gifts of Fast Black Salt K, and to Dr W. S. Bauld, Dr E. Diczfalusy and Dr L. L. Engel for sending us details of their work before publication.

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The Nature of Collagen-Chondroitin Sulphate Linkages in Tendon

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In previous communications it has been suggested that chondroitin sulphate plays an important part in stabilizing the structure of tendon (Jackson, 1953; Jackson & Ball, 1953). What little evidence is available suggests that the mucopolysaccharide is firmly linked to the collagen of tendon (Meyer, 1952). Einbinder & Schubert (1951) found that the uptake of chondroitin sulphate by purified tendon collagen was maximal at pH 3 and minimal in the physiological pH range, and on this evidence they suggested that the linkage is not salt-like. Mörner (1889) held that chondroitin sulphate occurred in cartilage as an alkali-metal salt, while Meyer, Palmer & Smyth (1937) were of the view that cartilage contains a salt of chondroitin sulphate and collagen. Partridge (1948) favours an explanation half-way between these two extremes, and considers that the strongly acidic sulphate groups are held in combination with some of the basic protein residues, the net charge of the complex being adjusted mainly by competition between alkali-metal ions and hydrogen ions for carboxylic acid residues in both protein and polysaccharide. Woodin (1952) considers that in cornea the collagen-mucopolysaccharide linkages are similar to those between adjacent collagen polypeptide chains.

Mammalian collagen is generally considered to be stabilized by (1) electrovalencies located at polar side chains, forming salt-like cross-linkages, and (2) co-ordinate valencies between adjacent peptide groups (hydrogen bonds) (Gustavson, 1942*a*).

Astbury (1940) has stated that anything which interferes with the interaction of any interchain linkage must inevitably influence the thermal transformation temperature. Theis (1946) concludes that the shrinkage temperature may be defined as the specific point at which disruptive tendencies exceed the cohesive forces, thus making this temperature an actual measurement of the structural stability of collagen expressed in temperature units.

Further, it has been pointed out that an evaluation of the relative importance of the two types of directed valency present in tendon, can be obtained by measuring the shrinkage temperature of collagen after treatment specific for these two linkages (Gustavson, 1949*a*). β-Naphthalenesulphonic acid will inactivate the basic groups completely, eliminating the salt-like cross-linkages, without swelling the collagen and without affecting the co-ordinative activity of the peptide groups (Gustavson, 1942*a*). Using this reagent he has shown that the shrinkage temperature of skin is reduced by 10–12°, whereas urea, generally expected to break hydrogen bonds, lowers this temperature by about 40°. This means that hydrogen-bond formation is the more important in determining fibre stability. Strong solutions of calcium chloride have the same effect as urea (Theis, 1946; Partridge, 1948), dilute solutions also effecting some reduction in shrinkage temperature (Theis, 1946).

The effect of concentrated calcium chloride solutions is considered to be due to an interaction of the neutral salt with hydrogen bonds, causing a weakening and rupture of these stabilizing links (Gustavson, 1926), while an ionic interaction probably predominates in dilute solutions (Gustavson, 1949*a*). For these reasons shrinkage temperatures were determined in a wide range of calcium chloride concentrations (0–2·7 M), as a measure of the remaining linkages after treatment with the various reagents used.

Periodic acid oxidizes 1:2 glycol and 1-amino-2-hydroxy groups with the formation of aldehyde groups and was introduced as a method of splitting carbon-carbon bonds by Malaprade (1928, 1934). A preliminary experiment showed that periodic acid oxidation was effective with chondroitin sulphate. If the hydroxyl groups of chondroitin sulphate are concerned with hydrogen-bond formation in tendon, periodic acid oxidation of the hydroxyl groups should lead to a reduction in shrinkage temperature.

In this communication the results of applying these methods to rat-tail tendon and reconstituted collagen are reported. The results obtained have been interpreted as confirming previous suggestions concerning the role of chondroitin sulphate in stabilizing tendon (Jackson, 1953; Jackson & Ball, 1953) and as pointing to both salt-like cross-linkages and to hydrogen bonding as the forces concerned in the collagen-chondroitin sulphate association. The presence in tendon of a further stabilizing mucoprotein is also suggested.

EXPERIMENTAL METHODS

Materials

Tendon was obtained from the tails of freshly killed rats, cut into 1 cm. lengths and stored in the frozen state. Care was taken not to damage the fibres, any damaged ones being rejected.

Reconstituted collagen was prepared by precipitation from a solution of calf tendo Achillis in 0·4% acetic acid with 10% (w/v) NaCl and with chondroitin sulphate solution (1 mg./ml.) as previously described (Jackson, 1953). The precipitate consisted of a loose network of fine fibrils of varying dimensions, which made it difficult to obtain samples of uniform size. However, samples approximately 1 cm. in length were used for the shrinkage temperature determinations. Salt-precipitated collagen will be referred to as S.P.C., collagen precipitated by chondroitin sulphate as C.P.C.

Treatment of materials

Hyaluronidase. The method used was that described in the previous communication (Jackson, 1953).

β-Naphthalenesulphonic acid. Samples of 0·5 g. of each preparation was soaked in 5% (w/v) β-naphthalenesulphonic acid (pH 1·2) for 24 hr., and washed thoroughly with distilled water. The excess of acid was titrated with 0·2N-NaOH using methyl orange as indicator; the amount of acid fixed was determined by difference. 0·86–0·90 m-equiv. of acid were fixed by all the preparations. Microscopic examination of the tendons after treatment revealed no evidence of swelling, although similar treatment with HCl at the same pH (1·2) caused considerable swelling and destruction of the fibre structure.

Periodic acid. The reagent was prepared by dissolving 0·5 g. $NaIO_4$, $3H_2O$ and 0·5 ml. 70% (v/v) HNO_3 in 100 ml. water. This solution has a pH of 2·5, and in order to suppress the swelling of collagen which would occur at this pH, NaCl was added to a concentration of 2 M. The material was incubated in this solution at 25° for 24 hr. Similar reaction mixtures, with HCl at pH 2·5 in place of the periodic acid, were set up as controls. After treatment, all materials were washed thoroughly with distilled water.

Shrinkage temperature determinations

These were carried out in the apparatus previously described (Jackson, 1953). After being subjected to the appropriate treatment, the shrinkage temperature determinations were carried out in triplicate in increasing concentrations of $CaCl_2$ (0–2·7 M). The temperature was increased rapidly up to 5° below the shrinkage temperature and then very slowly in steps of 1° and held at each step for 2 min. Little or no swelling was apparent during the determinations but the specimen became translucent at about 8–10° below the actual shrinkage temperature. Preliminary experiments with native tendon and S.P.C. indicated that no pH change occurred in the media during the shrinkage temperature determinations. Preliminary determinations made on twenty specimens of S.P.C. and of tendon gave, for S.P.C. 42°±(S.D. 1°); for tendon 66°±(S.D. 1°). Shrinkage temperature was plotted against concentration of $CaCl_2$.

RESULTS

Untreated material. The results are shown in Fig. 1. The curve for native tendon is similar to that obtained by Theis (1946). There was first a rapid fall in shrinkage temperature with $CaCl_2$ concentrations up to 0·3 M, followed by a slower fall. Collapse of the collagen structure at room temperature occurred at 2·4 M-$CaCl_2$. Treatment of tendon with hyaluronidase reduced the shrinkage temperature in water by 12° to 55°. The shrinkage temperature remained at this level up to 0·8 M-$CaCl_2$, so that there was no initial rapid fall. The shrinkage temperature of enzyme-treated tendon continued higher than that of untreated tendon from 0·1 M to 1·5 M-$CaCl_2$. The curve of C.P.C. followed that of untreated tendon at a lower level and, similarly, the curve of S.P.C. was parallel to that of enzyme-treated tendon; the fall in shrinkage temperature of S.P.C. was more rapid than that of the other three preparations, complete structural collapse at room temperature occurring at 1·5 M-$CaCl_2$ as compared to 2·1–2·4 M.

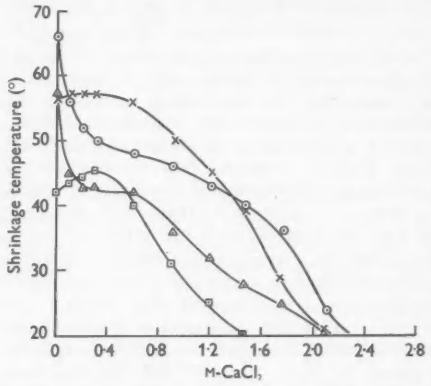

Fig. 1. Shrinkage temperature in $CaCl_2$. ⊙—⊙, rat-tail tendon; ×—×, rat-tail tendon treated with hyaluronidase (Jackson, 1953); △—△, chondroitin sulphate-precipitated collagen; ⊡—⊡, salt-precipitated collagen.

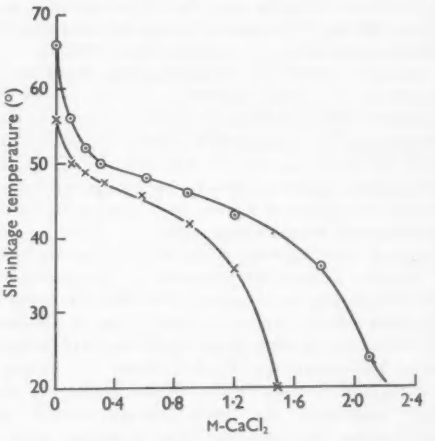

Fig. 2. Shrinkage temperature in $CaCl_2$ after treatment with 5% β-naphthalenesulphonic acid: ⊙—⊙, untreated tendon; ×—×, treated tendon.

After treatment with β-naphthalenesulphonic acid. The results are shown in Fig. 2. The decrease of 10° in the shrinkage temperature of native tendon in water was similar to that reported for skin collagen by Gustavson (1942*a*). The initial rapid fall in shrinkage temperature shown by untreated tendon was reduced by this treatment from 14° to about 8°. There was also a more rapid fall from 0·3 M-$CaCl_2$ onwards, room-temperature shrinkage occurring at 1·5 M-$CaCl_2$ after treatment, compared with 2·4 M-$CaCl_2$ before treatment. The shrinkage temperature in water of hyaluronidase-treated tendon was reduced by a further 4° (from 55 to 51°).

After treatment, S.P.C. formed a sticky, glue-like mass and became extremely swollen during washing with distilled water and disintegrated rapidly. The material dissolved in 0·9 % (w/v) NaCl, and increasing the salt concentration to 5 % (w/v) NaCl caused a precipitate to appear which was found to be fibrous. C.P.C. also formed a glue-like mass, which, on washing with water, disintegrated to a fine suspension but was insoluble in 0·9 % (w/v) NaCl. Increasing the salt concentration to 5 % (w/v) NaCl caused the suspension to coagulate into a granular precipitate in which no fibrous structure was visible under the optical microscope.

After treatment with β-naphthalenesulphonic acid, S.P.C. collapsed at room temperature in the most dilute calcium chloride, C.P.C. showing room-temperature shrinkage at 0·3 M-$CaCl_2$.

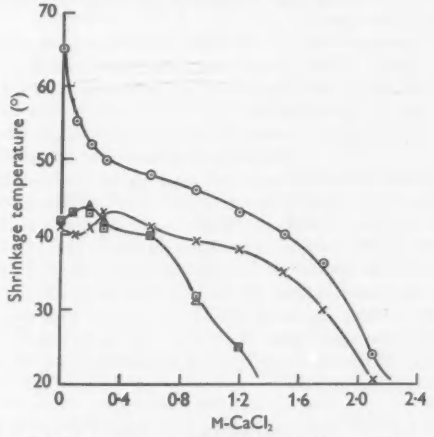

Fig. 3. Shrinkage temperature in $CaCl_2$ after periodic acid treatment; ⊙—⊙, rat-tail tendon treated with HCl (pH 2·5) containing 2 M-NaCl; ×—×, native tendon and hyaluronidase-treated tendon treated with 0·5 % (w/v) periodic acid containing 2 M-NaCl; ⊡—⊡, salt-precipitated collagen treated with periodic acid (pH 2·5) containing 2 M-NaCl; △—△, chondroitin sulphate-precipitated collagen treated with 0·5 % (w/v) periodic acid containing 2 M-NaCl.

After periodic acid treatment. The results are shown in Fig. 3. The effect of periodic acid treatment was quite striking. The shrinkage temperature of tendon in water was reduced by 24°, while that of hyaluronidase-treated tendon, which started some 12° lower, was reduced by 10–12°, so that the curves for enzyme-treated and native tendon start at the same level and are identical throughout. The initial rapid fall in shrinkage temperature was replaced by a slight rise, with a maximum at 0·4 M-$CaCl_2$, as compared with 0·2 M for S.P.C. Room temperature shrinkage of tendon occurred at 2·1 M-$CaCl_2$ as compared with 2·4 M-$CaCl_2$ before periodic acid treatment. This treatment had no effect on S.P.C., the two curves being identical before and after treatment, but treatment with periodic acid reduced the shrinkage temperature of C.P.C. in water to that of S.P.C., the two curves now being identical.

It was also noticed that tendon became considerably more swollen in water after treatment and split up into a mass of very fine fibrils.

Treatment of tendon with β-naphthalenesulphonic acid following periodic acid oxidation caused a further reduction of shrinkage temperature in water from 42 to 37°.

DISCUSSION

In calcium chloride, the shrinkage temperature curves of native tendon and C.P.C. are parallel, as are those of hyaluronidase-treated tendon and S.P.C. The essential difference between these two groups of curves lies mainly in the absence of an initial rapid fall of the shrinkage temperature at lower concentrations of $CaCl_2$ (0–0·3 M) (Fig. 1), in the latter group. These observations suggest that the first part of the shrinkage temperature curve of tendon relates to the chondroitin-collagen linkages. Theis (1946) interpreted this initial fall in shrinkage temperature in dilute calcium chloride as being due to the effect on ionic links between adjacent collagen polypeptide chains.

In view of the absence of swelling after treatment with β-naphthalenesulphonic acid, and the unchanged pH of the media during shrinkage temperature determinations, it seems likely that the hydrogen bonds between adjacent collagen-polypeptide chains remain unaffected and that the effect of this reagent is confined to the abolition of ionic bonds, as has been suggested by Gustavson (1942*a*). If this is so, then from Theis's interpretation, the initial fall in shrinkage temperature should not occur after treatment with β-naphthalenesulphonic acid, whereas this treatment reduces the initial fall by only 6° (from 14 to 8°) (Fig. 2). Moreover, the curve for S.P.C., which has been shown to contain salt-like cross-linkages (see below), fails to show the expected fall in shrinkage temperature in

dilute calcium chloride (Fig. 1). Thus the initial fall in shrinkage temperature may not be a function of intercollagen cross-links. In view of the effect of hyaluronidase on the shrinkage temperature curve for tendon, and of the absence of chondroitin sulphate in reconstituted collagen, an alternative interpretation is that the effect of low concentrations of calcium chloride on the shrinkage temperature is due to the splitting of chondroitin sulphate-collagen links, part of which are ionic, the remainder presumably being hydrogen bonds. This hydrogen bonding might take place through the hydroxyl groups of chondroitin sulphate and treatment with periodic acid, which would be expected to destroy these groups (Malaprade, 1928), does in fact abolish the initial fall in shrinkage temperature (Fig. 3). In this respect periodic acid is even more effective than hyaluronidase, the shrinkage temperature in water being reduced by 24° as compared with 10–12°. Periodic acid will oxidize the free hydroxyl-amino acids such as serine and threonine; but as the amino groups of these amino acids are involved in peptide links in proteins, it is unlikely that these amino acid residues in collagen polypeptide chains will be oxidized. Moreover, the shrinkage temperature curve of S.P.C. is unaffected by periodic acid treatment. The results with periodic acid on tendon may mean (*a*) that it is more destructive than hyaluronidase, or (*b*) that some other unsuspected stabilizing mucopolysaccharide or mucoprotein, unaffected by the enzyme, is present in tendon. Further study of the composition of tendon would be necessary before any decision could be made between these alternative explanations.

Treatment of native tendon with β-naphthalenesulphonic acid reduces its shrinkage temperature in water by 10°, whereas after hyaluronidase and periodic acid treatment, the same reagent produces a reduction in shrinkage temperature of only 4°. A possible interpretation of these facts would be that only part of the 10° reduction in shrinkage temperature is due to destruction by β-naphthalenesulphonic acid of ionic links between adjacent collagen chains, which would probably remain unaffected by hyaluronidase and periodic acid, and that these reagents affect the ionic links between mucopolysaccharide and collagen.

Complete structural collapse at room temperature of both hyaluronidase-treated and native tendon, occurs only in concentrated calcium chloride solutions, which indicates that the more important bonds stabilizing tendon are hydrogen bonds between adjacent collagen-polypeptide chains.

After periodic acid oxidation, room-temperature shrinkage occurs in 2·1 M-$CaCl_2$ as compared with 2·4 M-$CaCl_2$ for untreated tendon, which suggests again that bonds between adjacent collagen chains are not affected.

Some light is also thrown on the structure of salt-precipitated collagen by the effect of β-naphthalenesulphonic acid treatment. The complete structural collapse at room temperature in dilute calcium chloride, its solubility in 0·9% NaCl, and the swelling and disintegration in water all suggest that the stabilization of its structure is mainly by salt links, hydrogen bonds playing a very small part. Its behaviour is very similar to that of fish-skin collagen, which has a shrinkage temperature of 42° (Gustavson, 1942*b*) and forms a glue-like mass with α-lignosulphonic acid, which has a similar effect to β-naphthalenesulphonic acid (Gustavson, 1949*b*). Gustavson has interpreted this evidence as meaning that salt-like links mainly hold the structure together, the major proportion of the peptide groups not being internally compensated.

The observation that the salt-reconstituted collagen used in this work is soluble in 0·9% (w/v) NaCl, after treatment with β-naphthalenesulphonic acid, and can be reprecipitated from this solution in a fibrous form by increasing the salt concentration to 5% (w/v) NaCl, is interesting in view of the similar behaviour of collagen reconstituted from a collagen solution in 1·2% acetic acid, without previous treatment with β-naphthalenesulphonic acid (Jackson & Ball, 1953). This supports the suggestion then made that the end-to-end links in this material must be very weak, and suggests that these links involve only van der Waal's forces, since salt links are eliminated by β-naphthalenesulphonic acid.

The behaviour of C.P.C. after this treatment with β-naphthalenesulphonic acid provides further evidence of the function of chondroitin sulphate as a stabilizing factor.

In conclusion, if we accept Gustavson's suggestion that the effect on shrinkage temperature of various specific reagents may be used as a method of evaluating the relative importance of the two types of directed valency (Gustavson, 1949*a*), four-fifths of the stabilizing bonds in tendon are probably hydrogen bonds, the remainder being ionic cross-links. The linkages between chondroitin sulphate and collagen are probably of both types, with ionic links less important than the hydrogen bonding, together accounting for about a quarter of the total stabilizing bonds.

SUMMARY

1. The shrinkage temperature curves in calcium chloride of native rat-tail tendon, hyaluronidase-treated tendon, salt-precipitated and chondroitin sulphate-precipitated collagen have been determined before and after treatment with hyaluronidase, with β-naphthalenesulphonic acid and with periodic acid.

2. Chondroitin sulphate was found to play a part in the stabilization of tendon. It is suggested that a quarter of the stabilizing linkages are between chondroitin sulphate and collagen, consisting of 40 % salt-like links and 60 % hydrogen bonds.

3. Some other mucopolysaccharide or mucoprotein may also be concerned in this stabilization.

4. Salt-precipitated collagen is similar to fish skin in being stabilized mainly by salt-like cross-links.

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Chromatography of Pyruvic Acid and of Acetoacetic Acid as their 2:4-Dinitrophenylhydrazones

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The problem of the separation and the quantitative estimation of keto acids in the blood is of the greatest interest on account of the importance of these metabolic intermediates. Markees & Gey (1953) dealt with the quantitative paper chromatography of pyruvic acid as the 2:4-dinitrophenylhydrazone and showed that the derivatives of this α-keto acid form two sharply distinct spots with the R_F values of 0·36 (*A*) and 0·57 (*B*). This chromatographic separation has already been observed and described by other authors (Cavallini, Frontali & Toschi, 1949; LePage, 1950; Altmann, Crook & Datta, 1951; Seligson & Shapiro, 1952; Hockenhull, Hunter & Herbert, 1953; Stewart, 1953). The formation of these two spots *A* and *B* is most probably due to the formation of *cis* and *trans* isomerides.

The publication of El Hawary & Thompson (1953), in which the 2:4-dinitrophenylhydrazones of α-oxoglutaric acid, oxaloacetic acid, pyruvic acid, acetoacetic acid and acetone have been investigated chromatographically, prompted us to a renewed investigation of this problem. They found on analysis of their chromatograms a spot in the R_F region of 0·46–0·64 which they believed to be due to acetoacetic acid. Experimentally they proceeded in a very similar manner to ourselves, with the difference that we used ethyl acetate as a solvent for adding the substances to the chromatogram, while they, instead, employed sodium hydroxide plus phosphate buffer. The developing solvent was identical in both cases (butanol:ammonia:water) except that they added 10 %(v/v) ethanol which we did not. The question now arises whether the spot in the R_F region of 0·46–0·64 can be considered as due to the 2:4-dinitrophenylhydrazone of acetoacetic acid, as has been suggested by El Hawary & Thompson (1953), or whether it has possibly been mistaken by them for the spot which we call *B*, and which appears to be due to a reaction product of 2:4-dinitrophenylhydrazine with pyruvic acid.

EXPERIMENTAL AND RESULTS

In order to clear up this point we spotted the chemically prepared 2:4-dinitrophenylhydrazone of acetoacetic acid directly, and we also carried out an analysis of blood to which acetoacetic acid had been added. In one case we also boiled the blood filtrate before the addition of 2:4-dinitrophenylhydrazine, and, in another case, we analysed it without boiling. The experimental details were exactly the same as described in our earlier work (Markees & Gey, 1953). Controls using buffered sodium hydroxide as the solvent did not show any change in the R_F values of the various 2:4-dinitrophenylhydrazones. The results of these experiments can be summarized as follows:

Chemically prepared acetoacetic acid 2:4-dinitrophenylhydrazone gives a spot with an R_F value of 0·48. We also find a spot with the R_F value of 0·94 which corresponds to the acetone hydrazone formed by decomposition.

Chemically prepared pyruvic acid 2:4-dinitrophenylhydrazone always forms the above-mentioned spots *A* and *B*. The R_F value of 0·57 of *B* is quite different from that of the dinitrophenylhydrazone of acetoacetic acid.

When substance *A*, isolated by extracting chromatograms, is spotted along with acetoacetic acid 2:4-dinitrophenylhydrazone, two spots are obtained which run into each other, thus forming a streak. A similar thing happens when the 2:4-dinitrophenylhydrazone of acetoacetic acid is combined with the extract of spot *B*, only in a correspondingly higher R_F region. When the chemically obtained pyruvic acid 2:4-dinitrophenylhydrazone ($A+B$) is added to acetoacetic acid 2:4-dinitrophenylhydrazone, a long, continuous, yellow streak stretching from *A* to *B* (Fig. 1) is formed.

Normal blood without any addition gives besides the spots *A* and *B* a spot for α-oxoglutaric acid with the R_F value 0·07, which can be disregarded. When acetoacetic acid is added to the blood, it is almost completely transformed by the analytical procedure into acetone, even if the blood filtrate is not boiled before the addition of the 2:4-dinitrophenylhydrazine, and the spot of acetone 2:4-dinitrophenylhydrazone appears in its usual position. The same results are obtained whether the blood is boiled or not. If the blood contains large amounts of acetoacetic acid (large addition or diabetic acidosis), a fainter spot located between *A* and *B* appears when the blood filtrate is used unboiled.

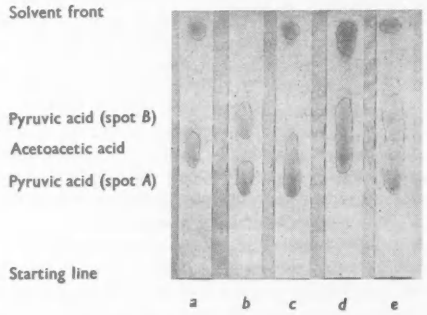

Fig. 1. Chromatograms of pure keto acid 2:4-dinitrophenylhydrazones. *a*, acetoacetic acid 2:4-dinitrophenylhydrazone ($R_F=0·48$); (*b*) pyruvic acid 2:4-dinitrophenylhydrazone (spot *A*, $R_F=0·36$; spot *B*, $R_F=0·57$); *c*, substance *A* + acetoacetic acid 2:4-dinitrophenylhydrazone; *d*, substance *B* + acetoacetic acid 2:4-dinitrophenylhydrazone; *e*, substances *A* + *B* + acetoacetic acid 2:4-dinitrophenylhydrazone.

DISCUSSION

From our experimental results we conclude that in the ordinary chromatography of the 2:4-dinitrophenylhydrazones of the keto acids in blood, that of acetoacetic acid only appears if the latter is in great excess. The 2:4-dinitrophenylhydrazone of acetoacetic acid has an R_F value of 0·48 and is sharply separated from the spot *B* of pyruvic acid which has an R_F value of 0·57. El Hawary & Thompson (1953), like ourselves, observed that the acetoacetic acid 2:4-dinitrophenylhydrazone spot almost completely disappears, owing to the analytical procedure, and that in its place acetone dinitrophenylhydrazone appears. Moreover, they were unable to obtain absorption curves identical with those of the chemically pure acetoacetic acid hydrazone with an extract of the spot which they ascribed to the acetoacetic acid hydrazone. We believe it probable that these authors have mistaken the spot *B* due to the pyruvic acid 2:4-dinitrophenylhydrazone for the acetoacetic acid 2:4-dinitrophenylhydrazone.

In a recent publication of Neish (1953), concerning the separation of the dinitrophenylhydrazones of the α-keto acids by ionophoresis and their polarographic estimation, there is also evidence that two spots may be found with the dinitrophenylhydrazone of pyruvic acid. The author says that this chromatographic separation is only found when old solutions are chromatographed and that with the freshly prepared dinitrophenylhydrazone of pyruvic acid only one spot appears. He explains this phenomenon as the formation of two isomers. With absolutely freshly prepared 2:4-dinitrophenylhydrazone of purest pyruvic acid, we have also observed a strong preponderance of fraction *A*. In the blood the relative intensity of the two spots is liable to marked variations, but complete absence of *B* has never been observed.

SUMMARY

1. Paper chromatography of the 2:4-dinitrophenylhydrazone of pyruvic acid shows two sharply separated spots with R_F values 0·36 (*A*) and 0·57 (*B*).

2. Acetoacetic acid dinitrophenylhydrazone has an R_F value of 0·48 and is only formed in the ordinary analytical procedure when acetoacetic acid is present in high concentration in the blood. In most cases, owing to decomposition, acetone 2:4-dinitrophenylhydrazone with the R_F value 0·94 is formed exclusively.

3. Attention is directed to the possibility of error in mistaking the spot *B* due to pyruvic acid for the spot due to acetoacetic acid.

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Index of Authors

Note. Entries marked with an asterisk (*) refer to communications made in title only.
Entries marked with a section mark (§) refer to contributions to Symposia.
Small roman numerals refer to pages in *Proceedings of the Biochemical Society.*

ABDULLAH, M. M., MORCOS, S. R. & SALAH, M. K. Content of vitamin A_2 in some Nile fishes 569

ADAMS, D. H. Liver catalase activity in the riboflavin deficient mouse xxvi

ADJUTANTIS, G. Isolation of cellular constituents using a combination of the Behrens and the Schneider techniques xlii

ALDRIDGE, W. N. Tricresyl phosphates and cholinesterase 185

ANDERSON, A. J. A method for the isolation of urinary mucoproteins xxv

ASATOOR, A. M. & KING, E. J. Simplified colorimetric blood sugar method xliv

ASHBY, J. H., CROOK, E. M. & DATTA, S. P. Thermodynamic quantities for the dissociation equilibria of biologically important compounds. 1. Theory and experimental method 190

ASHBY, J. H., CROOK, E. M. & DATTA, S. P. Thermodynamic quantities for the dissociation equilibria of biologically important compounds. 2. The acid dissociations of glycerol 2-phosphoric acid 198

ASKONAS, B. A., CAMPBELL, P. N., HUMPHREY, J. H. & WORK, T. S. The source of antibody globulin in rabbit milk and goat colostrum 597

ASKONAS, B. A., CAMPBELL, P. N. & WORK, T. S. The distribution of radioactivity in goat casein after injection of radioactive amino acids and its bearing on theories of protein synthesis iv

BADDILEY, J., HUGHES, D. E., MATHIAS, A. P. & PIERPOINT, W. S. The phosphorylation of pantothenic acid by *Lactobacillus arabinosus* 17–5 xxii

BALMAIN, J. H., FOLLEY, S. J. & GLASCOCK, R. F. Relative utilization of glucose and acetate carbon for lipogenesis by mammary-gland slices, studied with tritium, ^{13}C and ^{14}C 234

BALMAIN, J. H., FOLLEY, S. J., GLASCOCK, R. F. & MCNAUGHT, M. L. Effects of adrenal corticoids on lipogenesis in mammary tissue *in vitro* vi

BARTLEY, W. Metabolism of thiamine phosphates in washed suspensions of kidney particles 379

BARTLEY, W. The formation of phosphopyruvate by washed suspensions of sheep kidney particles 387

BAULD, W. S. Some errors in the colorimetric estimation of oestriol, oestrone and oestradiol by the Kober reaction 426

BENTOR, V. *see* WERTHEIMER, E. 297

BERGMANN, E. D., LITTAUER, U. Z. & VOLCANI, B. E. The formation of D-3-phosphoglyceric acid from pentoses by *Escherichia coli* 147

BHATIA, I. S. *see* SRINIVASAN, M. 256

BICKEL, H. *see* BOSCOTT, R. J. i

BILLETT, F. S. *see* CORNER, E. D. S. 270

BILLING, B. H. The quantitative determination of bile pigments in serum using reverse phase partition chromatography xxx

BIRD, R. & HOPKINS, R. H. The action of some α-amylases on amylose 86

BIRD, R. & HOPKINS, R. H. The mechanism of β-amylase action. 2. 'Multichain' action on amylose fission products 140

BLAIR, J. A. & GRAHAM, J. The pigments of snake skins. 1. The isolation of riboflavin as a pigment of the skins of the green snakes *Philothamnus semivariegatus* and *Dispholidus typus* 286

BLAKLEY, R. L. Folic acid as coenzyme for the interconversion of serine and glycine xvii

BOAG, J. W. *see* VAN HEYNINGEN, R. 372

BONNER, W. D. jun. Activation of the succinic dehydrogenase-cytochrome system 274

BOSCOTT, R. J. & BICKEL, H. Phenylalanine and tyrosine metabolism in patients with phenylketonuria i

BOURNE, G. H. A histochemical study of the specificity of phosphatases xxi, *xxvii

BOWDEN, C. H. & MACLAGAN, N. F. The detection of thyroxine and related substances on paper chromatograms by the ceric sulphate-arsenious acid reaction vii

BOWDEN, C. H. *see also* WILKINSON, J. H. 215

BOYLAND, E., WALLACE, D. M. & WILLIAMS, D. C. The activity of the enzymes sulphatase and glucuronidase in the urine of normal and cancer patients xxix

BRADISH, C. J. & BROOKSBY, J. B. Electrophoretic studies of ox serum. 3. The sera of cattle infected with the virus of vesicular stomatitis 342

BRADISH, C. J., HENDERSON, W. M. & BROOKSBY, J. B. Electrophoretic studies of ox serum. 1. The sera of normal cattle 329

BRADISH, C. J., HENDERSON, W. M. & BROOKSBY, J. B. Electrophoretic studies of ox serum. 2. The sera of cattle infected with the virus of foot-and-mouth disease 335

BRADLEY, J. E. S. Apparatus for simultaneously measuring ultraviolet adsorption and radioactivity in the eluate from chromatographic columns xlviii

BRADLEY, J. E. S. Automatic recording scanner for isotope-bearing chromatograms xlviii

BROADBENT, I. E. & KLYNE, W. The specificity of the dinitrobenzene reaction for ketosteroids xxx

BROOKSBY, J. B. *see* BRADISH, C. J. 329, 335, 342

BROWN, F. & JACKSON, H. A simple technique for the estimation of radioactive components of plasma after the administration of radioactive iodide 399

*BROWN, F. B., GANT, D. E., SMITH, E. LESTER & PARKER, L. F. J. Paper electrophoresis and chromatography of substances related to vitamin B_{12} xlix

BROWN, F. B. & SMITH, E. LESTER. New purines in B_{12} vitamins xxxiv

BROWNIE, A. C. & GRANT, J. K. Enzymic 11-β-hydroxylation of progesterone by ox adrenocortical mitochondria xxv

CAMPBELL, P. N. *see* ASKONAS, B. A. 597, iv

CASPARY, E. A. & KEKWICK, R. A. Observations on the molecular weight of human fibrinogen xxxv

CHALLENGER, F., LEAVER, D. & WHITAKER, M. I. Alkyl sulphides evolved from dog's urine by sodium hydroxide ii

CHALMERS, J. G. Heat transformation products of cottonseed oil 487

CHANDA, R., CLAPHAM, H. M. & OWEN, E. C. Dietary carotene and the degree of esterification of vitamin A in the milk and blood of cows 453

CHARLWOOD, P. A. Sedimentation and diffusion of human albumins. 3. Various normal human albumin preparations 259

CHARLWOOD, P. A. Sedimentation and diffusion of human albumins. 4. Low concentration behaviour of serum albumins in cases of liver disease 480
CHEESMAN, D. F. The effect of nucleotoxic stilbene derivatives on globin monolayers xlii
CHEN, Y.-T. *see* ISHERWOOD, F. A. 1, 15; MAPSON, L. W. 21
CHUGHTAI, I. D. & WALKER, T. K. The mechanism of the formation of organic acids by mould fungi. 6. The influence of cyanide on *Aspergillus niger* 484
CLAPHAM, H. M. *see* CHANDA, R. 453
CLARKE, H. B., CUSWORTH, D. C. & DATTA, S. P. The dissociation constants of the magnesium salts of phosphoric acid, glucose 1-phosphoric acid and glycerol 2-phosphoric acid xiv
COLLINS, F. D., GREEN, J. N. & MORTON, R. A. Studies in rhodopsin. 7. Regeneration of rhodopsin by comminuted ox retina 493
COMFORT, A. The effect of plasma β-lipoprotein concentration on the action of injected heparin in the rabbit ix
COMFORT, A. & WEATHERALL, M. Porphyrins in the urine of normal men xix,* xxvii
CORNER, E. D. S., BILLETT, F. S. & YOUNG, L. Biochemical studies of toxic agents. 6. The conversion of naphthalene into 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid in the rabbit 270
CROOK, E. M., HARRIS, H., HASSAN, F. & WARREN, F. L. Continuous direct photometry of dyed materials in filter paper with special reference to the estimation of proteins separated by electrophoresis 434
*CROOK, E. M., HARRIS, H., HASSAN, F. & WARREN, F. L. Photometry of dyed material in filter paper xxviii
CROOK, E. M. *see also* ASHBY, J. H. 190, 198
CROWTHER, S., FULTON, J. D. & JOYNER, L. P. The metabolism of *Leishmania donovani* in culture 182
CUSWORTH, D. C. *see* CLARKE, H. B. xiv

DALGLIESH, C. E. & TEKMAN, S. The excretion of kynurenine and 3-hydroxykynurenine by man 458
DALZIEL, K. & O'BRIEN, J. R. P. Spectrophotometric studies of the reaction of methaemoglobin with hydrogen peroxide. 1. The formation of methaemoglobin-hydrogen peroxide 648
DALZIEL, K. & O'BRIEN, J. R. P. Spectrophotometric studies of the reaction of methaemoglobin with hydrogen peroxide. 2. The degradation of methaemoglobin by hydrogen peroxide 660
DATTA, S. P. *see* ASHBY, J. H. 190, 198; CLARKE, H. B. xiv
DAVIES, D. A. L., MORGAN, W. T. J. & MOSIMANN, W. Studies in immunochemistry. 13. Preparation and properties of the 'O' somatic antigen of *Shigella dysenteriae* (Shiga) 572
DAVIES, J. T. Some factors influencing the orientation of ϵ-amino groups in monolayers of proteins and amino acid polymers 509
DAVIES, R. E. *see* KORNBERG, H. L. 355, 363; MITCHELL, F. L. 690; WHITTAM, R. 445
DAWSON, R. M. C. A note on the estimation of sphingomyelin in nervous tissue 621
DENT, C. E. & FOWLER, D. I. Paper-chromatographic analysis of Dakin's samples of 'β-hydroxyglutamic acid' 54
DESIKACHAR, H. S. R. & MCHENRY, E. W. Some effects of vitamin B_6 deficiency on fat metabolism in rats 544
DODGSON, K. S., MELVILLE, T. H., SPENCER, B. & WILLIAMS, K. The arylsulphatase of an *Alcaligenes* isolated from a marine mud xxiv
DODGSON, K. S. & SPENCER, B. An examination of human serum for the presence of arylsulphatase xiii
DODGSON, K. S., SPENCER, B. & THOMAS, J. Studies on sulphatases. 6. The localization of arylsulphatase in the rat-liver cell 177
DRESEL, E. I. B. & FALK, J. E. Studies on the biosynthesis of blood pigments. 1. Haem synthesis in haemolysed erythrocytes of chicken blood 156
DUTTON, R. W. A simplified apparatus for the photosynthesis of [^{14}C] starch and sugars xlviii

EDWARDS, R. W. H. & KELLIE, A. E. Micro-estimation of 'formaldehydrogenic steroids' by bismuthate oxidation 207
EGGLESTON, L. V. & WILLIAMSON, D. H. The turnover rates of the phosphate groups of flavin-adenine dinucleotide and adenosine triphosphate during oxidative phosphorylation 250
ENNOR, A. H. & ROSENBERG, H. An investigation into the turnover rates of organophosphates. 1. Extracellular space and intracellular inorganic phosphate in skeletal muscle 302
ENNOR, A. H. & ROSENBERG, H. An investigation into the turnover rates of organophosphates. 2. The rate of incorporation of ^{32}P into adenosine triphosphate and phosphocreatine in skeletal muscle 308
EVANS, G. G. & REITH, W. S. Studies on the determination of the sequence of amino acids in peptides and proteins. 3. The synthesis of 3-(4′-dimethylamino-3′:5′-dinitrophenyl)hydantoin derivatives of various amino acids, and their use for the determination of *N*-terminal amino acids 111

FALK, J. E. *see* DRESEL, E. I. B. 156
FILDES, J. E., LAURENCE, D. J. R. & REES, V. H. The dye-binding capacity of human plasma determined fluorimetrically, and its relation to the determination of plasma albumin xxxi
FLETCHER, A. P. The measurement of the components of the plasminogen-plasmin system in biological fluids 677
FLETCHER, C. M., LOWTHER, A. G. & REITH, W. S. Studies on the determination of the sequence of amino acids in peptides and proteins. 2. The separation of the methyl esters of *N*-2:4-dinitrophenyl derivatives of amino acids by adsorption chromatography and the free amino groups of insulin 106
FLETCHER, E. T. D. *see* SCOTT BLAIR, G. W. 504
FOLLEY, S. J. *see* BALMAIN, J. H. 234, vi
FORD, J. E. & HOLDSWORTH, E. S. Biosynthetic relationships between vitamin B_{12}-like factors xxxv
FOWLER, D. I. *see* DENT, C. E. 54
FULTON, J. D. *see* CROWTHER, S. 182

GALLOP, R. C. *see* SMITH, H. ix
GANT, D. E., SMITH, E. LESTER & PARKER, L. F. J. Removal of nucleotide from B_{12} vitamins xxxiv
*GANT, D. E. *see also* BROWN, F. B. xlix
GEORGE, P. & HANANIA, G. I. H. pH Changes accompanying the formation of metmyoglobin complexes xxxvii
GEORGE, P. & HANANIA, G. I. H. The mechanism of formation of metmyoglobin complexes xxxviii
GILLILAND, I. C., HANNO, M. G. & STRUDWICK, J. I. Protein-bound polysaccharides in diabetes with and without complications xxxii
GLASCOCK, R. F. *see* BALMAIN, J. H. 234, vi
GLOCK, G. E. & MCLEAN, P. Levels of enzymes of the direct oxidative pathway of carbohydrate metabolism in mammalian tissues and tumours 171
GOODWIN, T. W. & OSMAN, H. G. Studies in carotenogenesis. 10. Spirilloxanthin synthesis by washed cells of *Rhodospirillum rubrum* 222
GOODWIN, T. W. & PENDLINGTON, S. The biosynthesis of riboflavin in *Eremothecium ashbyii* xxiv
GRAHAM, J. *see* BLAIR, J. A. 286
GRANT, J. K. *see* BROWNIE, A. C. xxv
GRANT, R. A. Light scattering apparatus xxvii

GREEN, J. The 'anti-thrombic' action of heparin; the relation of sulphydryl groups xxi
GREEN, J. N. *see* COLLINS, F. D. 493
GREENBAUM, A. L. & GREENWOOD, F. C. Some enzymic changes in the mammary gland of rats during pregnancy, lactation and mammary involution 625
GREENWOOD, F. C. *see* GREENBAUM, A. L. 625

HAGEN, P. The distribution of ascorbic acid between the particulate and non-particulate components of adrenal and liver cells 44
HAHN, H. H. *see* SILK, M. H. 406
HAMER, D. Interaction of acids of high molecular weight with albumin 610
HANANIA, G. I. H. *see* GEORGE, P. xxxvii, xxxviii
HANNO, M. G. *see* GILLILAND, I. C. xxxii
HARKNESS, R. D., MARKO, A. M., MUIR, H. M. & NEUBERGER, A. The metabolism of collagen and other proteins of the skin of rabbits 558
*HARRIS, H., MITTWOCH, U., ROBSON, E. B. & WARREN, F. L. Genetics of cystinuria xxviii
HARRIS, H. *see also* CROOK, E. M. 434, *xxviii
HARTLES, R. L. & WASDELL, M. R. The metabolism of the oral flora. 2. The oxidation of some sugars by mixed human saliva 353
HARTLEY, B. S. & KILBY, B. A. The reaction of *p*-nitrophenyl esters with chymotrypsin and insulin 288
HASLEWOOD, G. A. D. Comparative studies of 'bile salts'. 7. Bile acids of the coypu, *Myocastor coypus* 581
HASLEWOOD, G. A. D. 'Hyocholic acid', a trihydroxy bile acid from pig bile xxxviii
HASSAN, F. *see* CROOK, E. M. 434, *xxviii
HAWKINS, J. B. & YOUNG, L. Biochemical studies of toxic agents. 5. Observations on the fate of ^{35}S-labelled arylsulphuric acids following their administration to the rat 166
HEALD, P. J. Rapid changes in cerebral creatine phosphate xxxix
HEALD, P. J., MCILWAIN, H. & NELLIGAN, D. Apparatus for maintaining and rapidly transferring sliced tissues xlviii
HENDERSON, W. M. *see* BRADISH, C. J. 329, 335
HENRY, K. M. *see* MCNAUGHT, M. L. 151
HEPPEL, L. A. & WHITFELD, P. R. Enzymic synthesis of alkyl esters of ribomononucleotides ii
HEPPEL, L. A., WHITFELD, P. R. & MARKHAM, R. Exchange reactions involving dinucleotides and cyclic mononucleotides iii
HERBERT, M. *see* HOCKENHULL, D. J. D. 73
HESTRIN, S. *see* SCHRAMM, M. 163
HIRD, F. J. R. & SPRINGELL, P. H. The enzymic reaction of amino acids with glutathione 417
HOCKENHULL, D. J. D., HERBERT, M., WALKER, A. D., WILKIN, G. D. & WINDER, F. G. Organic acid metabolism of *Penicillium chrysogenum*. 1. Lactate and acetate 73
HOLDSWORTH, E. S. *see* FORD, J. E. xxxv
HOLMAN, W. I. M. A colorimetric method for the determination of the principal metabolites of nicotinic acid in human urine 513
HOLTON, F. A. *see* SLATER, E. C. 28
HOPKINS, R. H. & JELINEK, B. The mechanism of β-amylase action. 1. 'Multichain' action on amylose 136
HOPKINS, R. H. *see also* BIRD, R. 86, 140
HOWARD, B. H. & RAISTRICK, H. Studies in the biochemistry of micro-organisms. 91. The colouring matters of *Penicillium islandicum* Sopp. Part 3. Skyrin and flavoskyrin 56
HUGHES, D. E. *see* BADDILEY, J. xxii; PIERPOINT, W. S. 130
HUMPHREY, J. H. *see* ASKONAS, B. A. 597
HUNTER, G., SMITH, H. V. & TAYLOR, L. M. On the bromide test of permeability of the barrier between blood and cerebrospinal fluid—an assessment 588

ISHERWOOD, F. A., CHEN, Y.-T. & MAPSON, L. W. Isolation of D-glyceric acid from cress seedlings and its relationship to the synthesis of L-ascorbic acid 15
ISHERWOOD, F. A., CHEN, Y.-T. & MAPSON, L. W. Synthesis of L-ascorbic acid in plants and animals 1
ISHERWOOD, F. A. *see also* MAPSON, L. W. 21

JACKSON, D. S. Stabilizing linkages in tendon xv
JACKSON, D. S. The nature of collagen-chondroitin sulphate linkages in tendon 699
JACKSON, H. *see* BROWN, F. 399
JAYASURIYA, G. C. N. Citrate accumulation from glycollate by an oxalate decomposing organism xli
JELINEK, B. *see* HOPKINS, R. H. 136
JERMYN, M. A. & THOMAS, R. Multiple components in horse-radish peroxidase 631
JONES, N. R. Taurine in fresh and iced skeletal muscle of codling (*Gadus callarias*) xxii
JOYNER, L. P. *see* CROWTHER, S. 182

*KAWERAU, E. Electrophoresis in agar with the bridge unit xviii
KEKWICK, R. A. *see* CASPARY, E. A. xxxv
KELLIE, A. E. *see* EDWARDS, R. W. H. 207
KEMBLE, A. R. & MACPHERSON, H. T. Determination of monoamino monocarboxylic acids by quantitative paper chromatography 548
KEMP, A. & KITS VAN HEIJNINGEN, A. J. M. A colorimetric micro-method for the determination of glycogen in tissues 646
KEMP, A. *see also* MENDEL, B. 639
KENCH, J. E. Bile pigment formation *in vitro* from haematin and haem derivatives 669
KILBY, B. A. *see* HARTLEY, B. S. 288
KIND, P. R. N., KING, E. J., PASH, V., ROMAN, W. & SCHMIDT, E. Inhibition of enzymes by silicic acid xlv
KING, E. J. & PASH, V. Estimation of salicylates in blood plasma xlv
KING, E. J., PASH, V. & WEEDON, W. Colorimetric estimation of silica without removal of phosphate and iron xlvi
KING, E. J., STACY, B. D. & WEEDON, W. Colorimetric estimation of silica in urine xlvi
KING, E. J. *see also* ASATOOR, A. M. xliv; KIND, P. R. N. xlv; STACY, B. D. xxxiii
KITS VAN HEIJNINGEN, A. J. M. *see* KEMP, A. 646
KLECZKOWSKI, A. Stability of chymotrypsin and tobacco mosaic virus decreased by ultraviolet radiation 345
KLYNE, W. *see* BROADBENT, I. E. xxx
KNIVETT, V. A. Phosphorylation coupled with anaerobic breakdown of citrulline 602
KNIVETT, V. A. The effect of arsenate on bacterial citrulline breakdown 606
KON, S. K. *see* MCNAUGHT, M. L. 151
KORNBERG, H. L., DAVIES, R. E. & WOOD, D. R. The activity and function of gastric urease in the cat 363
KORNBERG, H. L., DAVIES, R. E. & WOOD, D. R. The breakdown of urea in cats not secreting gastric juice 355

LACEY, B. W. Variation of the antigenic structure of certain bacteria with temperature of incubation and ionic composition of the medium xiv
LASER, H. Intramanometric dialysis xx
LASER, H. Peroxidatic activity of catalase xx
LAURENCE, D. J. R. Automatic scanning of paper electrophoretic results xlv
LAURENCE, D. J. R. *see also* FILDES, J. E. xxxi; WEBER, G. xxxi
LAWSON, A. & SEARLE, C. E. Antithyroid activity of some *S*-substituted 2-mercaptoglyoxalines x
LEA, C. H. & RHODES, D. N. Phospholipids. 2. Estimation of amino nitrogen in intact phospholipids 613
LEAVER, D. *see* CHALLENGER, F. ii

LEWIS, D. The reduction of sulphate in the rumen of the sheep 391
LEWIS, G. M. & MACFARLANE, M. G. Examination of the effect of *Clostridium oedematiens* toxin on certain enzyme systems v
LEWIS, P., WIGGINS, H. S. & WOOTTON, I. D. P. A microcuvette for infrared spectrography xliii
LITTAUER, U. Z. *see* BERGMANN, E. D. 147
LOVELOCK, J. E. The protective action of neutral solutes against haemolysis by freezing and thawing 265
LOWTHER, A. G. *see* FLETCHER, C. M. 106

MCDONALD, I. W. The extent of conversion of food protein to microbial protein in the rumen of the sheep 120
MACFARLANE, M. G. *see* LEWIS, G. M. v
MCHENRY, E. W. *see* DESIKACHAR, H. S. R. 544
MCILWAIN, H. & RODNIGHT, R. Metabolism of mammalian tissues with little or no added aqueous phase xii
MCILWAIN, H. *see also* HEALD, P. J. xlviii; RODNIGHT, R. xii
MACINTYRE, I. The determination of serum calcium with a flame spectrophotometer xliii
MACINTYRE, I. & RALSTON, M. Direct determination of serum cholesterol xliii
MACLAGAN, N. F. & WILKINSON, J. H. The biological action of substances related to thyroxine. 7. The metabolism of butyl 4-hydroxy-3:5-diiodobenzoate 211
MACLAGAN, N. F. *see also* BOWDEN, C. H. vii; WILKINSON, J. H. 215, vii
MCLEAN, P. *see* GLOCK, G. E. 171
MCNAUGHT, M. L., OWEN, E. C., HENRY, K. M. & KON, S. K. The utilization of non-protein nitrogen in the bovine rumen. 8. The nutritive value of the proteins of preparations of dried rumen bacteria, rumen protozoa and brewer's yeast for rats 151
MCNAUGHT, M. L. *see also* BALMAIN, J. H. vi
MACPHERSON, H. T. *see* KEMBLE, A. R. 548
MAPSON, L. W., ISHERWOOD, F. A. & CHEN, Y.-T. Biological synthesis of L-ascorbic acid: the conversion of L-galactono-γ-lactone into L-ascorbic acid by plant mitochondria 21
MAPSON, L. W. *see also* ISHERWOOD, F. A. 1, 15
MARGOLIASH, E. The chromatographic behaviour of cytochrome *c* on cation exchangers 535
MARGOLIASH, E. The use of ion exchangers in the preparation and purification of cytochrome *c* 529
MARKEES, S. Chromatography of pyruvic acid and of acetoacetic acid as their 2:4-dinitrophenylhydrazones 703
MARKHAM, R. *see* HEPPEL, L. A. iii
MARKHAM, R. L. *see* SCOTT BLAIR, G. W. 504
MARKO, A. M. *see* HARKNESS, R. D. 558
MATHIAS, A. P. *see* BADDILEY, J. xxii
MELVILLE, T. H. *see* DODGSON, K. S. xxiv
MENDEL, B., KEMP, A. & MYERS, D. K. A colorimetric micro-method for the determination of glucose 639
MILLS, G. L. Specificity of bond fission during the acid hydrolysis of insulin 230
MITCHELL, F. L. & DAVIES, R. E. The isolation and estimation of the steroid oestrogens in placental tissue 690
*MITTWOCH, U. *see* HARRIS, H. xxviii
MORCOS, S. R. *see* ABDULLAH, M. M. 569
MORELL, D. B. Derivatives and kinetics of lactoperoxidase 683
MORGAN, D. M. *see* WADE, H. E. 41
MORGAN, W. T. J. *see* DAVIES, D. A. L. 572
MORRISON, J. F. Studies on aconitase xxxvi
MORRISON, J. F. The purification of aconitase 99
MORRISON, J. F. & PETERS, R. A. The inhibition of aconitase by fluorocitrate xxxvi
MORTON, R. A. *see* COLLINS, F. D. 493
MOSIMANN, W. *see* DAVIES, D. A. L. 572
MUIR, H. M. *see* HARKNESS, R. D. 558

MYERS, C. M. & SMITH, J. N. Comparative detoxication. 2. Glucoside formation from phenols in locusts 498
MYERS, D. K. *see* MENDEL, B. 639

NEIL, M. W. & WALKER, D. G. Paper ionophoresis of phosphoric esters xxvii
NELLIGAN, D. *see* HEALD, P. J. xlviii
NEUBERGER, A. *see* HARKNESS, R. D. 558
NEUMARK, E. Gaucher's disease; a rare disorder of lipoid storage xviii
NEWTON, B. A. The use of a compound forming fluorescent conjugates with protein in studying the mode of action of polymyxin xxxii
NICOLA, M. DE The carotenoids of the carapace of the echinoderm *Ophidiaster ophidianus* 555

O'BRIEN, J. R. P. *see* DAIZIEL, K. 648, 660
OSMAN, H. G. *see* GOODWIN, T. W. 222
OWEN, E. C. *see* CHANDA, R. 453; MCNAUGHT, M. L. 151

PARKE, D. V. The occurrence of lactose in the spleen cerebrosides of a case of Gaucher's disease xv
PARKER, L. F. J. *see* BROWN, F. B. *xlix; GANT, D. E. xxxiv
PARR, C. W. The separation of sugars and of sugar phosphates by gradient elution from ion-exchange columns xxvii
PASH, V. *see* KIND, P. R. N. xlv; King, E. J. xlv, xlvi
PENDLINGTON, S. *see* GOODWIN, T. W. xxiv
PENNINGTON, R. J. The metabolism of short-chain fatty acids in the sheep. 2. Further studies with rumen epithelium 410
PETERS, R. A. *see* MORRISON, J. F. xxxvi
PIERPOINT, W. S. & HUGHES, D. E. The synthesis of coenzyme A by *Lactobacillus arabinosus* 17–5 130
PIERPOINT, W. S. *see also* BADDILEY, J. xxii
PIRIE, A. *see* VAN HEYNINGEN, R. 372
PIRIE, N. W. The fission of tobacco mosaic virus and some other nucleoproteins by strontium nitrate 83
PLEASE, N. W. Estimation of the variance of the data used in the calculation of dissociation constants. *Appendix to* Thermodynamic quantities for the dissociation equilibria of biologically important compounds. 1. Theory and experimental method (Ashby, J. H., Crook, E. M. & Datta, S. P.) 196
POPJÁK, G. & TIETZ, A. Biosynthesis of fatty acids by slices and cell-free suspensions of mammary gland 46
POPJÁK, G. & TIETZ, A. Synthesis of fatty acids from acetate by preparation of the 'cell-sap' of rat mammary gland xxiii
POSTGATE, J. R. Presence of cytochrome in an obligate anaerobe xi
PRATT, O. E. The dephosphorylation of thiamine pyrophosphate by suspensions of cerebral tissues iii

RAISTRICK, H. *see* HOWARD, B. H. 56
RALSTON, M. *see* MACINTYRE, I. xliii
REES, V. H. *see* FILDES, J. E. xxxi
REITH, W. S. & WALDRON, N. M. Studies on the determination of the sequence of amino acids in peptides and proteins. 4. The synthesis of 3-(4′-dimethylamino-3′:5′-dinitrophenyl)-2-thiohydantoin derivatives of various amino acids, and their use for amino acid-sequence determinations 116
REITH, W. S. *see also* EVANS, G. G. 111; FLETCHER, C. M. 106
RHODES, D. N. *see* LEA, C. H. 613
ROBINSON, D., SMITH, J. N. & WILLIAMS, R. T. The metabolism of *iso*propylbenzene (cumene) xi
*ROBSON, E. B. *see* HARRIS, H. xxviii
RODNIGHT, R. & MCILWAIN, H. Metabolism of mammalian tissue-fragments suspended in non-aqueous fluids xii
RODNIGHT, R. *see also* MCILWAIN, H. xii

ROMAN, W. *see* KIND, P. R. N. xlv
ROSENBERG, H. *see* ENNOR, A. H. 302, 308

SALAH, M. K. *see* ABDULLAH, M. M. 569
SARMA, P. S. *see* SUNDARARAJAN, T. A. 125
SCHMIDT, E. *see* KIND, P. R. N. xlv
SCHOLES, G. & WEISS, J. Chemical action of X-rays on nucleic acids and related substances in aqueous systems. 2. The mechanism of the action of X-rays on nucleic acids in aqueous systems 65
SCHRAMM, M. & HESTRIN, S. Synthesis of cellulose by *Acetobacter xylinum*. 1. Micromethod for the determination of celluloses 163
SCOTT BLAIR, G. W., WILLIAMS, P. O., FLETCHER, E. T. D. & MARKHAM, R. L. On the flow of certain pathological human synovial effusions through narrow tubes 504
SEARLE, C. E. *see* LAWSON, A. x
SHERRATT, H. S. A. & THOMAS, A. J. The isolation of plant nucleic acid fractions for purine and pyrimidine determinations xli
SILK, M. H. & HAHN, H. H. The resolution of mixtures of C_{16}–C_{24} normal-chain fatty acids by reversed-phase partition chromatography 406
SLATER, E. C. & HOLTON, F. A. Oxidative phosphorylation coupled with the oxidation of α-ketoglutarate by heart-muscle sarcosomes. 2. Phosphorus:oxygen ratio 28
SMITH, E. LESTER *see* BROWN, F. B. xxxiv, *xlix; GANT, D. E. xxxiv
SMITH, H. & ZWARTOUW, H. T. Fractionation of the intracellular material of *Bacillus anthracis* grown *in vivo*: the polysaccharide and polyglutamic acid viii
SMITH, H., ZWARTOUW, H. T. & GALLOP, R. C. The nature of the aggressins produced by *Bacillus anthracis* growing *in vivo* ix
SMITH, H. V. *see* HUNTER, G. 588
SMITH, J. N., SMITHIES, R. H. & WILLIAMS, R. T. Studies in detoxication. 55. The metabolism of alkylbenzenes. (*a*) Glucuronic acid excretion following the administration of alkylbenzenes. (*b*) Elimination of toluene in the expired air of rabbits 317
SMITH, J. N., SMITHIES, R. H. & WILLIAMS, R. T. Studies in detoxication. 56. The metabolism of alkylbenzenes. Stereochemical aspects of the biological hydroxylation of ethylbenzene to methylphenylcarbinol 320
SMITH, J. N. & WILLIAMS, R. T. Studies in detoxication. 57. The structure of the glucuronides from 2- and 4-quinolones and the orientation of biological hydroxylation in quinolones 325
SMITH, J. N. & WILLIAMS, R. T. Studies in detoxication. 58. The metabolism of aliphatic alcohols. The glucuronic acid conjugation of chlorinated and some unsaturated alcohols 618
SMITH, J. N. *see also* MYERS, C. M. 498; ROBINSON, D. xi
SMITH, R. H. The phosphatides of the latex of *Hevea brasiliensis*. 1. Biochemical changes 240
SMITHIES, R. H. *see* SMITH, J. N. 317, 320
SOMOGYI, J. C. A spectrophotometric method for the determination of changes in the thiamine molecule xl
SPENCER, B. *see* DOGSON, K. S. 177, xiii, xxiv
SPRINGELL, P. H. *see* HIRD, F. J. R. 417
SPROTT, W. E. *see* WILKINSON, J. H. 215
SRINIVASAN, M. & BHATIA, I. S. The carbohydrates of *Agave vera cruz* Mill. 2. Distribution in the stem and pole 256
STACY, B. D. The colorimetric estimation of aluminium xlvii
STACY, B. D. The estimation of collagen in silicotic tissues xlvii
STACY, B. D. & KING, E. J. Silica and collagen in the lungs and lymph nodes of cortisone-treated silicotic rats xxxiii
STACY, B. D. *see also* KING, E. J. xlvi
STRUDWICK, J. I. *see* GILLILAND, I. C. xxxii
SUNDARARAJAN, T. A. & SARMA, P. S. Purification and properties of phosphoprotein phosphatase from ox spleen 125
SYNGE, R. L. M. & WOOD, J. C. Diffusible peptide-like and glycosidic constituents of Italian rye grass xix

TAYLOR, L. M. *see* HUNTER, G. 588
TAYLOR, W. The metabolism of progesterone by animal tissues *in vitro*. 1. Factors influencing the metabolism of progesterone by rat liver and the investigation of the products of metabolism 463
TEKMAN, S. *see* DALGLIESH, C. E. 458
TERNER, C. The effect of *p*-nitrophenol on the Pasteur reaction and on aerobic phosphorylation in suspensions of the mammary gland 471
THOMAS, A. J. *see* SHERRATT, H. S. A. xli
THOMAS, J. *see* DODGSON, K. S. 177
THOMAS, R. *see* JERMYN, M. A. 631
TIETZ, A. *see* POPJÁK, G. 46, xxiii

VAN HEYNINGEN, R., PIRIE, A. & BOAG, J. W. Changes in lens during the formation of X-ray cataract in rabbits 2. 372
VOLCANI, B. E. *see* BERGMANN, E. D. 147

WADE, H. E. & MORGAN, D. M. The analysis of adenosine triphosphate and adenosine diphosphate preparations by paper ionophoresis 41
WALDRON, N. M. *see* REITH, W. S. 116
WALKER, A. D. *see* HOCKENHULL, D. J. D. 73
WALKER, D. G. *see* NEIL, M. W. xxvii
WALKER, T. K. *see* CHUGHTAI, I. D. 484
WALLACE, D. M. *see* BOYLAND, E. xxix
WARREN, F. L. *see* CROOK, E. M. 434, *xxviii; HARRIS, H. *xxviii
WASDELL, M. R. *see* HARTLES, R. L. 353
WAYMOUTH, C. Some effects of inorganic phosphate and bicarbonate on cell survival and proliferation in chemically defined nutrient media iv
WEATHERALL, M. *see* COMFORT, A. xix, *xxvii
WEBER, G. & LAURENCE, D. J. R. Fluorescent indicators of adsorption in aqueous solution and on the solid phase xxxi
WEEDON, W. *see* KING, E. J. xlvi
WEIL-MALHERBE, H. Carbohydrate metabolism and plasma adrenaline level xxix
WEISS, J. *see* SCHOLES, G. 65
WERTHEIMER, E., BENTOR, V. & WURZEL, M. *In vitro* demonstration of metabolic changes during heat regulation in rats 297
WHITAKER, M. I. *see* CHALLENGER, F. ii
WHITFELD, P. R. *see* HEPPEL, L. A. ii, iii
WHITTAM, R. & DAVIES, R. E. Relations between metabolism and the rate of turnover of sodium and potassium in guinea pig kidney-cortex slices 445
WIGGINS, H. S. Conjugated bile acids of some primate biles xxxix
WIGGINS, H. S. *see also* LEWIS, P. xliii
WILKIN, G. D. *see* HOCKENHULL, D. J. D. 73
WILKINSON, J. H. & MACLAGAN, N. F. The effect of an anti-thyroxine compound on the deiodination of triiodothyronine in rats vii
WILKINSON, J. H., SPROTT, W. E., BOWDEN, C. H. & MACLAGAN, N. F. The biological action of substances related to thyroxine. 8. The effects of butyl 4-hydroxy-3:5-diiodobenzoate on the deiodination of diiodotyrosine and thyroxine in rats 215
WILKINSON, J. H. *see also* MACLAGAN, N. F. 211
WILLIAMS, D. C. *see* BOYLAND, E. xxix
WILLIAMS, K. *see* DODGSON, K. S. xxiv
WILLIAMS, P. O. *see* SCOTT BLAIR, G. W. 504

WILLIAMS, R. T. *see* ROBINSON, D. xi; SMITH, J. N. 317, 320, 325, 618
WILLIAMSON, D. H. *see* EGGLESTON, L. V. 250
WILSON, T. H. Concentration gradients of lactate, hydrogen and some other ions across the intestine *in vitro* 521
WINDER, F. G. *see* HOCKENHULL, D. J. D. 73
WOOD, D. R. *see* KORNBERG, H. L. 355, 363
WOOD, J. C. *see* SYNGE, R. L. M. xix
WOODHOUSE, D. L. The purine and pyrimidine composition of some deoxyribonucleic acids from tumours 349
WOOLF, L. I. The sugar-containing lipids of Gaucher's disease xvi
WOOTTON, I. D. P. Infrared spectra of a series of bile acids xxxix
WOOTTON, I. D. P. *see also* LEWIS, P. xliii
WORK, T. S. *see* ASKONAS, B. A. 597, iv
WURZEL, M. *see* WERTHEIMER, E. 297

YOUNG, L. *see* CORNER, E. D. S. 270; HAWKINS, J. B. 166

ZWARTOUW, H. T. *see* SMITH, H. viii, ix

Index of Subjects

Note. Entries marked with an asterisk (*) refer to communications made in title only.
Entries marked with a section mark (§) refer to contributions to Symposia.
Small roman numerals refer to pages in *Proceedings of the Biochemical Society.*

Acetate and glucose carbon, utilization for lipogenesis by mammary-gland slices (Balmain, J. H., Folley, S. J. & Glascock, R. F.) 234

Acetate, biosynthesis of fatty acids from, in mammary gland (Popják, G. & Tietz, A.) 46

Acetate, fatty acids from, synthesis by preparation of 'cell-sap' of rat mammary gland (Popják, & Tietz, A.) xxiii

Acetate in metabolism of *Penicillium chrysogenum* (Hockenhull, D. J. D., Herbert, M., Walker, A. D., Wilkin, G. D. & Winder, F. G.) 73

Acetoacetic acid 2:4-dinitrophenylhydrazone, chromatography of (Markees, S.) 703

Acetobacter xylinum, synthesis of cellulose by (Schramm, M. & Hestrin, S.) 163

Acetophenone, metabolism of, glucuronide formation from, in rabbits (Smith, J. N., Smithies, R. H. & Williams, R. T.) 320

p-Acetylphenylsulphate, synthesis and breakdown of, by rat-liver extracts (Dodgson, K. S., Spencer, B. & Thomas, J.) 177

Acid hydrolysis of insulin, specificity of bond fission during (Mills, G. L.) 230

Aconitase, inhibition by fluorocitrate (Morrison, J. F. & Peters, R. A.) xxxvi

Aconitase, purification of (Morrison, J. F.) 99

Aconitase, studies on (Morrison, J. F.) xxxvi

Adenosine phosphates, microdetermination by paper ionophoresis (Wade, H. E. & Morgan, D. M.) 41

Adenosine triphosphate, rate of incorporation of ^{32}P into, in skeletal muscle (Ennor, A. H. & Rosenberg, H.) 308

Adenosine triphosphate, turnover rates of phosphate groups of, during oxidative phosphorylation (Eggleston, L. V. & Williamson, D. H.) 250

Adrenal cells, ascorbic acid distribution in (Hagen, P.) 44

Adrenal corticoids, effect on lipogenesis in mammary tissue *in vitro* (Balmain, J. H., Folley, S. J., Glascock, R. F. & McNaught, M. L.) vi

Adrenaline level in plasma (Weil-Malherbe, H.) xxix

Adrenocortical mitochondria, ox, enzymic 11-β-hydroxylation of progesterone by (Brownie, A. C. & Grant, J. K.) xxv

Adsorption in aqueous solution and on solid phase, fluorescent indicators of (Weber, G. & Laurence, D. J. R.) xxxi

Agave vera cruz Mill., carbohydrates of (Srinivasan, M. & Bhatia, I. S.) 256

Aggressins produced by *Bacillus anthracis* growing *in vivo*, nature of (Smith, H., Zwartouw, H. T. & Gallop, R. C.) ix

Albumin, plasma, determination of, and dye-binding capacity of (Fildes, J. E., Laurence, D. J. R. & Rees, V. H.) xxxi

Albumins, human, sedimentation, diffusion and molecular weight of (Charlwood, P. A.) 259

Albumins, serum, in cases of liver disease (Charlwood, P. A.) 480

Alcaligenes, arylsulphatase of (Dodgson, K. S., Melville, T. H., Spencer, B. & Williams, K.) xxiv

Alcohols, aliphatic, detoxication of (Smith, J. N. & Williams, R. T.) 618

Alcohols, chlorinated and unsaturated aliphatic, detoxication of (Smith, J. N. & Williams, R. T.) 618

Alginic acid, interaction with serum albumin (Hamer, D.) 610

Alkyl sulphides evolved from dog's urine by sodium hydroxide (Challenger, F., Leaver, D. & Whitaker, M. I.) ii

Aluminium, colorimetric estimation of (Stacy, B. D.) xlvii

Amides, colorimetric estimation of (Holman, W. I. M.) 513

Amino acid polymers and proteins, monolayers of, orientation of ϵ-amino groups in (Davies, J. T.) 509

Amino acids, coloured derivatives of, for end group determination in peptides (Evans, G. G. & Reith, W. S.) 111; (Reith, W. S. & Waldron, N. M.) 116

Amino acids, neutral, quantitative chromatographic determination (Kemble, A. R. & Macpherson, H. T.) 548

Amino acids, reaction with chloramine-T (Kemble, A. R. & Macpherson, H. T.) 548

Amino acids, sequence in proteins, determination of (Evans, G. G. & Reith, W. S.) 111; (Reith, W. S. & Waldron, N. M.) 116

ϵ-Amino groups, orientation of, in monolayers of proteins and amino acid polymers (Davies, J. T.) 509

m-Aminophenolglucoside, absorption spectra (Myers, C. M. & Smith, J. N.) 498

Ammonium ion, effect on metabolism of rumen epithelium (Pennington, R. J.) 410

Amphetamine, effect on glycogenolysis in rat diaphragm (Wertheimer, E., Bentor, V. & Wurzel, M.) 297

β-Amylase action, mechanism of (Bird, R. & Hopkins, R. H.) 140; (Hopkins, R. H. & Jelinek, B.) 136

α-Amylases, action on amylose (Bird, R. & Hopkins, R. H.) 86

Amylopectin, hydrolysis by α-amylases (Bird, R. & Hopkins, R. H.) 86

Amylose, action of some α-amylases on (Bird, R. & Hopkins, R. H.) 86

Amylose fission products during action of β-amylase (Bird, R. & Hopkins, R. H.) 140

Amylose, 'multichain' action on (Hopkins, R. H. & Jelinek, B.) 136

Anaerobe, obligate, cytochrome in (Postgate, J. R.) xi

Antibody globulin, source in milk and colostrum (Askonas, B. A., Campbell, P. N., Humphrey, J. H. & Work, T. S.) 597

Antigen, 'O' somatic, of *Shigella dysenteriae* (Shiga), preparation and properties of (Davies, D. A. L., Morgan, W. T. J. & Mosimann, W.) 572

Antigenic structure of bacteria, variation with temperature of incubation and ionic composition of the medium (Lacey, B. W.) xiv

D-Arabo-ascorbic acid, synthesis in cress seedlings and rats (Isherwood, F. A., Chen, Y.-T. & Mapson, L. W.) 1

Arsenate, effect on citrulline breakdown (Knivett, V. A.) 606

Arylsulphatase in human serum (Dodgson, K. S. & Spenser, B.) xiii

Arylsulphatase, localization in rat-liver cells (Dodgson, K. S., Spencer, B. & Thomas, J.) 177

Arylsulphatase of *Alcaligenes* (Dodgson, K. S., Melville, T. H., Spencer, B. & Williams, K.) xxiv

Arylsulphate, synthesis in rat liver (Dodgson, K. S., Spencer, B. & Thomas, J.) 177

Arylsulphuric acids, labelled with ^{35}S, fate of, in rat (Hawkins, J. B. & Young, L.) 166

L-Ascorbic acid, biological synthesis, by plant mitochondria (Mapson, L. W., Isherwood, F. A. & Chen, Y.-T.) 21

Ascorbic acid distribution in adrenal and liver cells (Hagen, P.) 44
Ascorbic acid, oxidation by cytochrome *c* (Margoliash, E.) 535
L-Ascorbic acid, synthesis in cress seedlings (Isherwood, F. A., Chen, Y.-T. & Mapson, L. W.) 15
L-Ascorbic acid, synthesis in plants and animals (Isherwood, F. A., Chen, Y.-T. & Mapson, L. W.) 1
Aspergillus niger, cyanide on (Chughtai, I. D. & Walker, T. K.) 484
Aspergillus oryzae, α-amylase, action on amylose (Bird, R. & Hopkins, R. H.) 86

Bacillus anthracis grown *in vivo*, fractionation of intracellular material of (Smith, H. & Zwartouw, H. T.) viii
Bacillus anthracis grown *in vivo*, nature of aggressins produced by (Smith, H., Zwartouw, H. T. & Gallop, R. C.) ix
Bacillus anthracis, polysaccharide and polyglutamic acid of (Smith, H. & Zwartouw, H. T.) viii
Bacillus subtilis, α-amylase, action on amylose (Bird, R. & Hopkins, R. H.) 86
Bacteria, variation of antigenic structure of, with temperature of incubation and ionic composition of the medium (Lacey, B. W.) xiv
Bacteriochlorophyll, production by *Rhodospirillum rubrum* (Goodwin, T. W. & Osman, H. G.) 222
Benzenes, alkylated glucuronide formation from, in rabbits (Smith, J. N., Smithies, R. H. & Williams, R. T.) 317
Bicarbonate, effect on cell survival in tissue culture (Waymouth, C.) iv
Bile acid, trihydroxy, from pig bile, 'hyocholic acid' (Haslewood, G. A. D.) xxxviii
Bile acids, conjugated, of primate biles (Wiggins, H. S.) xxxix
Bile acids, infrared spectra of (Wootton, I. D. P.) xxxix
Bile pigment formation *in vitro* from haematin and haem derivatives (Kench, J. E.) 669
Bile pigments in serum, quantitative determination using reverse-phase partition chromatography (Billing, B. H.) xxx
'Bile salts', comparative studies of (Haslewood, G. A. D.) 581
Blood of cows, dietary carotene and esterification of vitamin A in (Chanda, R., Clapham, H. M. & Owen, E. C.) 453
Blood sugar method, simplified colorimetric (Asatoor, A. M. & King, E. J.) xliv
Bromide, estimation in serum and cerebrospinal fluid (Hunter, G., Smith, H. V. & Taylor, L. M.) 588
Bromide test of permeability of blood-cerebrospinal fluid barrier (Hunter, G., Smith, H. V. & Taylor, L. M.) 588
Buffers of glycerol 2-phosphate (Ashby, J. H., Crook, E. M. & Datta, S. P.) 198
Butyl 4-hydroxy-3:5-diiodobenzoate, effects of, on deiodination of diiodotyrosine and thyroxine in rats (Wilkinson, J. H., Sprott, W. E., Bowden, C. H. & Maclagan, N. F.) 215
Butyl 4-hydroxy-3:5-diiodobenzoate, metabolism of (Maclagan, N. F. & Wilkinson, J. H.) 211

Calcium, serum, determination with flame spectrophotometer (MacIntyre, I.) xliii
Cancer patients, sulphatase and glucuronidase in urine of (Boyland, E., Wallace, D. M. & Williams, D. C.) xxix
Carbohydrate metabolism in plasma (Weil-Malherbe, H.) xxix
Carbohydrates of *Agave vera cruz* Mill. (Srinivasan, M. & Bhatia, I. S.) 256
Carcinogenic activity, lack of, in heated cottonseed oil (Chalmers, J. G.) 487
Carotene, dietary, and esterification of vitamin A in milk and blood of cows (Chanda, R., Clapham, H. M. & Owen, E. C.) 453
Carotenogenesis, studies in (Goodwin, T. W. & Osman, H. G.) 222
Carotenoids of fishes of Nile (Abdullah, M. M., Morcos, S. R. & Salah, M. K.) 569
Carotenoids of the shell of the echinoderm *Ophidiaster ophidianus* (Nicola, M. de) 555
Casein, peptides from partial hydrolysates of (Askonas, B. A., Campbell, P. N. & Work, T. S.) iv
Catalase, liver, activity in riboflavin deficient mouse (Adams, D. H.) xxvi
Catalase, peroxidatic activity of (Laser, H.) xx
Cataract, X-ray, in rabbits, changes in lens during formation of (Van Heyningen, R., Pirie, A. & Boag, J. W.) 372
Cation-exchange resins, cytochrome *c* on (Margoliash, E.) 529, 535
Cellular constituents, isolation using Behrens and Schneider techniques (Adjutantis, G.) xlii
Cellulose, bacterial, estimation of (Schramm, M. & Hestrin, S.) 163
Cellulose, solubilization and hydrolysis of (Schramm, M. & Hestrin, S.) 163
Cellulose, synthesis by *Acetobacter xylinum* (Schramm, M. & Hestrin, S.) 163
Cerebral tissues, suspensions of, dephosphorylation of thiamine pyrophosphate by (Pratt, O. E.) iii
Cerebrosides, spleen, with Gaucher's disease, lactose in (Parke, D. V.) xv
Cerebrospinal fluid, estimation of bromide in, and effect of permeability barrier (Hunter, G., Smith, H. V. & Taylor, L. M.) 588
Chenodeoxycholic acid in coypu bile (Haslewood, G. A. D.) 581
Chloramine-T, reaction with amino acids (Kemble, A. R. & Macpherson, H. T.) 548
Choleglobin and other green haems, formation of (Dalziel, K. & O'Brien, J. R. P.) 660
Cholesterol, serum, direct determination (MacIntyre, I. & Ralston, M.) xliii
Cholic acid in coypu bile (Haslewood, G. A. D.) 581
Cholinesterase and tricresyl phosphates (Aldridge, W. N.) 185
Chromatograms, isotope-bearing, automatic recording scanner for (Bradley, J. E. S.) xlviii
Chromatographic analyses of the fission products of hexasaccharide by crystalline β-amylase (Bird, R. & Hopkins, R. H.) 140
Chromatographic analysis of Dakin's samples of β-hydroxyglutamic acid on paper (Dent, C. E. & Fowler, D. I.) 54
Chromatographic columns, eluate from, apparatus for simultaneously measuring ultraviolet adsorption and radioactivity in (Bradley, J. E. S.) xlviii
Chromatographic methods in isolation of steroid oestrogens of placental tissue (Mitchell, F. L. & Davies, R. E.) 690
Chromatographic separation of flavin nucleotides from other phosphates (Eggleston, L. V. & Williamson, D. H.) 250
Chromatography of pyruvic acid and of acetoacetic acid as their 2:4-dinitrophenylhydrazones (Markees, S.) 703
Chromatography of some thiamine derivatives (Bartley, W.) 379
Chromatography, reverse-phase partition, quantitative determination of bile pigments in serum using (Billing, B. H.) xxx
Chromatography, reversed-phase partition, resolution of mixtures of C_{16}–C_{24} fatty acids by (Silk, M. H. & Hahn, H. H.) 406
Chymotrypsin, reaction of *p*-nitrophenyl esters with (Hartley, B. S. & Kilby, B. A.) 288
Chymotrypsin, stability of, decreased by ultraviolet radiation (Kleczkowski, A.) 345
Citrate accumulation from glycollate by oxalate decomposing organism (Jayasuriya, G. C. N.) xli

Citric acid, effect of cyanide on formation of, by *Aspergillus niger* (Chughtai, I. D. & Walker, T. K.) 484
Citrulline breakdown, effect of arsenate on (Knivett, V. A.) 606
Citrulline, breakdown of and phosphorylation (Knivett, V. A.) 602
Citrulline, breakdown of and synthesis of adenosine triphosphate (Knivett, V. A.) 602
Citrulline, colorimetric estimation of (Knivett, V. A.) 606
Clostridium oedematiens toxin, effect on certain enzyme systems (Lewis, G. M. & Macfarlane, M. G.) v
Codling (*Gadus callarias*), muscle of, taurine in (Jones, N. R.) xxii
Coenzyme A levels in rabbit lens in X-ray cataract (Van Heyningen, R., Pirie, A. & Boag, J. W.) 372
Coenzyme A, synthesis by *Lactobacillus arabinosus* 17–5 (Pierpoint, W. S. & Hughes, D. E.) 130
Collagen, estimation in silicotic tissues (Stacy, B. D.) xlvii
Collagen in lungs and lymph nodes of cortisone-treated silicotic rats (Stacy, B. D. & King, E. J.) xxxiii
Collagen, metabolism in skin of rabbits (Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A.) 558
Collagen-chondroitin sulphate linkages in tendon (Jackson, D. S.) 699
Colostrum, source of antibody globulin in (Askonas, B. A., Campbell, P. N., Humphrey, J. H. & Work, T. S.) 597
Corticoids, adrenal, effect on lipogenesis in mammary tissue *in vitro* (Balmain, J. H., Folley, S. J., Glascock, R. F. & McNaught, M. L.) vi
Cortisone-treated silicotic rats, silica and collagen in lungs and lymph nodes of (Stacy, B. D. & King, E. J.) xxxiii
Cottonseed oil, heated, effect when injected into mice (Chalmers, J. G.) 487
Cows, blood and milk of, dietary carotene and esterification of vitamin A in (Chanda, R., Clapham, H. M. & Owen, E. C.) 453
Coypu, *Myocastor coypus*, bile acids of (Haslewood, G. A. D.) 581
Creatine phosphate, cerebral, rapid changes in (Heald, P. J.) xxxix
Cress seedlings, D-glyceric acid from (Isherwood, F. A., Chen, Y.-T. & Mapson, L. W.) 15
Cress seedlings, synthesis of L-ascorbic acid in (Isherwood, F. A., Chen, Y.-T. & Mapson, L. W.) 1
Cumene, metabolism of (Robinson, D., Smith, J. N. & Williams, R. T.) xi
Cyanide influence on *Aspergillus niger* (Chughtai, I. D. & Walker, T. K.) 484
Cysteine, aconitase activation by (Morrison, J. F.) 99
Cystine, effect on coenzyme A formation in *Lactobacillus arabinosus* in pantothenate deficiency (Pierpoint, W. S. & Hughes, D. E.) 130
*Cystinuria, genetics of (Harris, H., Mittwoch, U., Robson, E. B. & Warren, F. L.) xxviii
Cytochrome *c*, effect of trichloroacetic acid on (Margoliash, E.) 535
Cytochrome *c*, preparation and purification (Margoliash, E.) 529
Cytochrome *c*, stability and purification (Margoliash, E.) 535
Cytochrome in an obligate anaerobe (Postgate, J. R.) xi

Denaturation of chymotrypsin and tobacco mosaic virus by heat (Kleczkowski, A.) 345
Deoxyribonucleic acid, interaction with serum albumin (Hamer, D.) 610
Deoxyribonucleic acids from tumours, purine and pyrimidine composition of (Woodhouse, D. L.) 349
Detoxication, studies in (Smith, J. N., Smithies, R. H. & Williams, R. T.) 317, 320; (Smith, J. N. & Williams, R. T.) 325
Dextran sulphate, interaction with serum albumin (Hamer, D.) 610
Dextrins, linear, hydrolysis by α-amylases (Bird, R. & Hopkins, R. H.) 86
Diabetes, protein-bound polysaccharides in (Gilliland, I. C., Hanno, M. G. & Strudwick, J. I.) xxxii
Diaphragm, rat, glycogen synthesis and glucose utilization, effect of temperature (Wertheimer, E., Bentor, V. & Wurzel, M.) 297
1:2-Dihydro-2-hydroxy-1-naphthyl glucosiduronic acid, as metabolite of naphthalene (Corner, E. D. S., Billett, F. S. & Young, L.) 270
Diiodotyrosine and thyroxine in rats, deiodination of, effects of butyl 4-hydroxy-3:5-diiodobenzoate on (Wilkinson, J. H., Sprott, W. E., Bowden, C. H. & Maclagan, N. F.) 215
Dimethylaminodinitrophenylhydantoins of amino acids and their use in determination of *N*-terminal amino acids (Evans, G. G. & Reith, W. S.) 111
Dimethylaminodinitrophenylthiohydantoin derivatives of amino acids and use in amino acid sequence determination (Reith, W. S. & Waldron, N. M.) 116
Dinitrobenzene reaction, specificity for ketosteroids (Broadbent, I. E. & Klyne, W.) xxx
Dinitrophenol, effect on turnover of sodium and potassium in guinea pig kidney-cortex slices (Whittam, R. & Davies, R. E.) 445
2:4-Dinitrophenylamino acids, esters of (Fletcher, C. M., Lowther, A. G. & Reith, W. S.) 106
2:4-Dinitrophenylhydrazones of acetoacetic acid and pyruvic acid, chromatography of (Markees, S.) 703
Diphenylamine, effect on spirilloxanthin production by *Rhodospirillum rubrum* (Goodwin, T. W. & Osman, H. G.) 222
Dissociation constants of magnesium salts of phosphoric acid, glucose 1-phosphoric acid and glycerol 2-phosphoric acid (Clarke, H. B., Cusworth, D. C. & Datta, S. P.) xiv
Dissociation equilibria of biologically important compounds (Ashby, J. H., Crook, E. M. & Datta, S. P.) 190, 198
Dithionite, reaction of methaemoglobin-hydrogen peroxide complex with (Dalziel, K. & O'Brien, J. R. P.) 648
Dithionite, reduction by rumen micro-organisms (Lewis, D.) 391
Dog's urine, alkyl sulphides evolved from, by sodium hydroxide (Challenger, F., Leaver, D. & Whitaker, M. I.) ii

Echinoderm *Ophidiaster ophidianus*, carotenoids of shell of (Nicola, M. de) 555
Egg albumin, amino acid analysis (Kemble, A. R. & Macpherson, H. T.) 548
*Electrophoresis in agar with the bridge unit (Kawerau, E.) xviii
Electrophoresis, paper, estimation of proteins in (Crook, E. M., Harris, H., Hassan, F. & Warren, F. L.) 434
Electrophoretic studies of ox serum (Bradish, C. J. & Brooksby, J. B.) 342; (Bradish, C. J., Henderson, W. M. & Brooksby, J. B.) 329, 335
Enzyme levels in mammary gland during pregnancy, lactation and mammary involution (Greenbaum, A. L. & Greenwood, F. C.) 625
Enzyme levels in rabbit lens in X-ray cataract (Van Heyningen, R., Pirie, A. & Boag, J. W.) 372
Enzymes in urine of normal and cancer patients (Boyland, E., Wallace, D. M. & Williams, D. C.) xxix
Eremothecium ashbyii, biosynthesis of riboflavin in (Goodwin, T. W. & Pendlington, S.) xxiv
Escherichia coli, formation of D-3-phosphoglyceric acid from pentoses by (Bergmann, E. D., Littauer, U. Z. & Volcani ,B. E.) 147

Ethylbenzene, metabolism of, glucuronide formation from, in rabbits (Smith, J. N., Smithies, R. H. & Williams, R. T.) 320
Extracellular space in skeletal muscle (Ennor, A. H. & Rosenberg, H.) 302

Fat metabolism in rats, effects of vitamin B_6 deficiency on (Desikachar, H. S. R. & McHenry, E. W.) 544
Fatty acid biosynthesis in mammary gland (Popják, G. & Tietz, A.) 46
Fatty acids, C_{16}–C_{24} mixtures, resolution by reversed-phase partition chromatography (Silk, M. H. & Hahn, H. H.) 406
Fatty acids from acetate, synthesis by preparation of 'cell-sap' of rat mammary gland (Popják, G. & Tietz, A.) xxiii
Fatty acids, short-chain, metabolism of (Pennington, R. J.) 410
Fibrinogen, human, molecular weight of (Caspary, E. A. & Kekwick, R. A.) xxxv
Ficus macrophylla, peroxidases of (Jermyn, M. A. & Thomas, R.) 631
Fish muscle, taurine in (Jones, N. R.) xxii
Fishes of Nile, vitamin A_2 and other carotenoids of (Abdullah, M. M., Morcos, S. R. & Salah, M. K.) 569
Flavin-adenine dinucleotide, turnover rates of phosphate groups of, during oxidative phosphorylation (Eggleston, L. V. & Williamson, D. H.) 250
Flavoskyrin, colouring matter from *Penicillium islandicum* Sopp. (Howard, B. H. & Raistrick, H.) 56
Fluorescent conjugates with protein and mode of action of polymyxin (Newton, B. A.) xxxii
Fluorescent indicators of adsorption in aqueous solution and on solid phase (Weber, G. & Laurence, D. J. R.) xxxi
Fluoride, inhibition of porphyrin synthesis in haemolysates and whole chicken blood (Dresel, E. I. B. & Falk, J. E.) 156
Fluorimetric methods for plasma albumin (Fildes, J. E., Laurence, D. J. R. & Rees, V. H.) xxxi
Fluorocitrate, inhibition of aconitase by (Morrison, J. F. & Peters, R. A.) xxxvi
Folic acid as coenzyme for interconversion of serine and glycine (Blakley, R. L.) xvii
Folic acid, stimulation of haem synthesis by (Dresel, E. I. B. & Falk, J. E.) 156
Food protein conversion to microbial protein in rumen of sheep (McDonald, I. W.) 120
Foot-and-mouth disease, sera of cattle infected with (Bradish, C. J., Henderson, W. M. & Brooksby, J. B.) 335
Formaldehyde, effect on chloramine-T reaction with amino acids (Kemble, A. R. & Macpherson, H. T.) 548
Formaldehydogenic steroids, micro-estimation by bismuthate oxidation (Edwards, R. W. H. & Kellie, A. E.) 207

Gadus callarias *see* Codling
L-Galactono-γ-lactone conversion to L-ascorbic acid by plant mitochondria (Mapson, L. W., Isherwood, F. A. & Chen, Y.-T.) 21
Gaucher's disease, lactose in spleen cerebrosides with (Parke, D. V.) xv
Gaucher's disease, lipoid storage in (Neumark, E.) xviii
Gaucher's disease, sugar-containing lipids of (Woolf, L. I.) xvi
Globin monolayers, effect of nucleotoxic stilbene derivatives on (Cheesman, D. F.) xlii
Gluconic acid, effect of cyanide on formation of, by *Aspergillus niger* (Chughtai, I. D. & Walker, T. K.) 484
Glucose and acetate carbon, utilization for lipogenesis by mammary-gland slices (Balmain, J. H., Folley, S. J. & Glascock, R. F.) 234
Glucose, colorimetric micro-method for determination of (Mendel, B., Kemp, A. & Myers, D. K.) 639
Glucose, determination of, in tissues (Kemp, A. & Kits van Heijningen, A. J. M.) 646
Glucose 6-phosphate dehydrogenase, in lactating mammary gland (Glock, G. E. & McLean, P.) 171
Glucose 6-phosphate dehydrogenase, in tissues (Glock, G. E. & McLean, P.) 171
Glucose 6-phosphate dehydrogenase, in tumours (Glock, G. E. & McLean, P.) 171
Glucose 1-phosphoric acid, dissociation constants of magnesium salts of (Clarke, H. B., Cusworth, D. C. & Datta, S. P.) xiv
Glucose, transport across intestine *in vitro* (Wilson, T. H.) 521
Glucoside formation in locusts (Myers, C. M. & Smith, J. N.) 498
Glucuronidase in urine of normal and cancer patients (Boyland, E., Wallace, D. M. & Williams, D. C.) xxix
Glucuronide excretion in rabbits after feeding alkylbenzenes (Smith, J. N., Smithies, R. H. & Williams, R. T.) 320
Glucuronides, excretion in rabbits after feeding quinolones (Smith, J. N. & Williams, R. T.) 325
Glucuronides of aliphatic alcohols, formation in detoxication (Smith, J. N. & Williams, R. T.) 618
Glutathione, enzymic reaction of amino acids with (Hird, F. J. R. & Springell, P. H.) 417
D-Glyceric acid from cress seedlings, and its relationship to synthesis of L-ascorbic acid (Isherwood, F. A., Chen, Y.-T. & Mapson, L. W.) 15
Glycerol 2-phosphate, acid dissociations of (Ashby, J. H., Crook, E. M. & Datta, S. P.) 198
Glycerol 2-phosphoric acid, dissociation constants of magnesium salts of (Clarke, H. B., Cusworth, D. C. & Datta, S. P.) xiv
Glycine and serine, interconversion of, folic acid as coenzyme for (Blakley, R. L.) xvii
Glycine, incorporation into haem by haemolysates of chicken blood (Dresel, E. I. B. & Falk, J. E.) 156
Glycogen in tissues, colorimetric micro-method for determination of (Kemp, A. & Kits van Heijningen, A. J. M.) 646
Glycogen synthesis and glucose utilization by abdominal muscle of mice previously exposed to cold (Wertheimer, E., Bentor, V. & Wurzel, M.) 297
Glycogen synthesis in rat diaphragm and liver, effect of temperature (Wertheimer, E., Bentor, V. & Wurzel, M.) 297
Glycosidic constituents, diffusible, of Italian rye grass (Synge, R. L. M. & Wood, J. C.) xix

Haematin and haem derivatives, bile pigment formation *in vitro* from (Kench, J. E.) 669
Haemolysis by freezing and thawing, protective action of neutral solutes against (Lovelock, J. E.) 365
Heart-muscle sarcosomes, oxidative phosphorylation in (Slater, E. C. & Holton, F. A.) 28
Heparin action and effect on plasma β-lipoprotein in rabbit (Comfort, A.) ix
Heparin, 'anti-thrombic' action of; relation of sulphydryl groups (Green, J.) xxi
Heparin, interaction with serum albumin (Hamer, D.) 610
Hevea brasiliensis, phosphorus and choline in phospholipids of latex of (Smith, R. H.) 240
Hexasaccharide fission products, chromatographic analyses by crystalline β-amylase (Bird, R. & Hopkins, R. H.) 140
Histochemical study of specificity of phosphatases (Bourne, G. H.) xxi, *xxvii
Horse-radish peroxidase, multiple components in (Jermyn, M. A. & Thomas, R.) 631
Hydrogen peroxide, degradation of methaemoglobin by (Dalziel, K. & O'Brien, J. R. P.) 660
Hydrogen peroxide methaemoglobin compound, breakdown of (Dalziel, K. & O'Brien, J. R. P.) 660

Hydrogen peroxide, spectrophotometric studies of reaction of methaemoglobin with (Dalziel, K. & O'Brien, J. R. P.) 648, 660
4-Hydroxy-3:5-diiodobenzoate, methylation of, in man (Maclagan, N. F. & Wilkinson, J. H.) 211
β-Hydroxyglutamic acid, Dakin's samples of, chromatographic analysis on paper (Dent, C. E. & Fowler, D. I.) 54
3-Hydroxykynurenine and kyurenine, excretion by man (Dalgliesh, C. E. & Tekman, S.) 458
'Hyocholic acid', a trihydroxy bile acid from pig bile (Haslewood, G. A. D.) xxxviii

Infrared spectrography, microcuvette for (Lewis, P., Wiggins, H. S. & Wootton, I. D. P.) xliii
Insulin, acid hydrolysis of, specificity of bond fission during (Mills, G. L.) 230
Insulin, end groups of (Fletcher, C. M., Lowther, A. G. & Reith, W. S.) 106
Insulin, loss activity in hydrolysis (Mills, G. L.) 230
Insulin, ox, amino acid analysis (Kemble, A. R. & Macpherson, H. T.) 548
Insulin, partial acid hydrolysis (Mills, G. L.) 230
Insulin, reaction of *p*-nitrophenyl esters with (Hartley, B. S. & Kilby, B. A.) 288
Intestine, concentration gradients of lactate and hydrogen ions across, *in vitro* (Wilson, T. H.) 521
Intracellular inorganic phosphate in skeletal muscle (Ennor, A. H. & Rosenberg, H.) 302
Intramanometric dialysis (Laser, H.) xx
Iodide and thyroxine, rapid quantitative determination of relative proportions of, after administration of radioactive iodide (Brown, F. & Jackson, H.) 399
Iodine, radioactive, effect in man and rats (Brown, F. & Jackson, H.) 399
Ion-exchange columns, gradient elution from, separation of sugars and sugar phosphates by (Parr, C. W.) xxvii
Ion-exchange resins in preparation of cytochrome *c* (Margoliash, E.) 529, 535
Ionophoresis, paper, in analysis of adenosine phosphates (Wade, H. E. & Morgan, D. M.) 41
Ions, transport across intestine *in vitro* (Wilson, T. H.) 521
Iron, aconitase activation by (Morrison, J. F.) 99

α-Ketoglutarate, oxidation, and phosphorylation in heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 28
Ketosteroids, specificity of dinitrobenzene reaction for (Broadbent, I. E. & Klyne, W.) xxx
Kidney particles, washed suspensions of, formation of phosphopyruvate by (Bartley, W.) 387
Kidney particles, washed suspensions of, metabolism of thiamine phosphates in (Bartley, W.) 379
Kidney-cortex slices, turnover of sodium and potassium in (Whittam, R. & Davies, R. E.) 445
Kynurenine and 3-hydroxykynurenine, excretion by man (Dalgliesh, C. E. & Tekman, S.) 458

Lactate in metabolism of *Penicillium chrysogenum* (Hockenhull, D. J. D., Herbert, M., Walker, A. D., Wilkin, G. D. & Winder, F. G.) 73
Lactate, transport across intestine *in vitro* (Wilson, T. H.) 521
Lactobacillus arabinosus, phosphorylation of pantothenic acid by (Baddiley, J., Hughes, D. E., Mathias, A. P. & Pierpoint, W. S.) xxii
Lactobacillus arabinosus 17–5, synthesis of coenzyme A by (Pierpoint, W. S. & Hughes, D. E.) 130
Lactoperoxidase, derivatives and kinetics of (Morell, D. B.) 683
Lactose in spleen cerebrosides with Gaucher's disease (Parke, D. V.) xv
Leishmania donovani in culture, metabolism of (Crowther, S., Fulton, J. D. & Joyner, L. P.) 182
Lens, changes in, during formation of X-ray cataract in rabbits (Van Heyningen, R., Pirie, A. & Boag, J. W.) 372
Light scattering apparatus (Grant, R. A.) xxvii
Lipids, sugar-containing, of Gaucher's disease (Woolf, L. I.) xvi
Lipogenesis in mammary tissue *in vitro*, adrenal corticoids on (Balmain, J. H., Folley, S. J., Glascock, R. F. & McNaught, M. L.) vi
Lipogenesis, utilization of glucose and acetate carbon for, by mammary-gland slices (Balmain, J. H., Folley, S. J. & Glascock, R. F.) 234
Lipoid storage in Gaucher's disease (Neumark, E.) xviii
β-Lipoprotein, plasma, effect on action of heparin in rabbit (Comfort, A.) ix
Lipothiamide, the reactive form of thiamine (Bartley, W.) 379
Liver cells, ascorbic acid distribution in (Hagen, P.) 44
Liver disease, serum albumins in (Charlwood, P. A.) 480
Liver, rat, glycogen synthesis and glucose utilization, effect of temperature (Wertheimer, E., Bentor, V. & Wurzel, M.) 297
Liver, steroid metabolism in suspensions of (Taylor, W.) 463
Locusts, detoxication in (Myers, C. M. & Smith, J. N.) 498

Malonate, inhibition of fatty acid metabolism in rumen epithelium (Pennington, R. J.) 410
Mammary gland cell-free suspensions, fatty acid synthesis by (Popják, G. & Tietz, A.) 46
Mammary gland, enzyme levels in, during pregnancy, lactation and mammary involution (Greenbaum, A. L. & Greenwood, F. G.) 625
Mammary gland, rat, preparation of 'cell-sap' of, synthesis of fatty acids from acetate by (Popják, G. & Tietz, A.) xxiii
Mammary-gland slices, fatty acid synthesis by (Popják, G. & Tietz, A.) 46
Mammary-gland slices, sheep and rat, relative utilization of glucose and acetate carbon for lipogenesis by (Balmain, J. H., Folley, S. J. & Glascock, R. F.) 234
Mammary gland, suspension of, effect of *p*-nitrophenol on Pasteur reaction and on aerobic phosphorylation in (Terner, C.) 471
Mammary tissue *in vitro*, adrenal corticoids on lipogenesis in (Balmain, J. H., Folley, S. J., Glascock, R. F. & McNaught, M. L.) vi
Media for tissue culture (Waymouth, C.) iv
Medium, effect on tissue metabolism (McIlwain, H. & Rodnight, R.) xii; (Rodnight, R. & McIlwain, H.) xii
Mercaptoglyoxalines, *S*-substituted, thyroid activity of (Lawson, A. & Searle, C. E.) x
Metabolism in guinea pig kidney-cortex slices and turnover of sodium and potassium (Whittam, R. & Davies, R. E.) 445
Methaemoglobin, degradation by hydrogen peroxide (Dalziel, K. & O'Brien, J. R. P.) 660
Methaemoglobin, spectrophotometric studies of reaction of, with hydrogen peroxide (Dalziel, K. & O'Brien, J. R. P.) 648, 660
Methaemoglobin-hydrogen peroxide, formation of (Dalziel, K. & O'Brien, J. R. P.) 648
2-Methyladenine from vitamin B_{12} (Brown, F. B. & Smith, E. Lester) xxxiv
Methylation of 4-hydroxy-3:5-diiodobenzoate in man (Maclagan, N. F. & Wilkinson, J. H.) 211
2-Methylhypoxanthine from vitamin B_{12} (Brown, F. B. & Smith, E. Lester) xxxiv
Methylphenylcarbinol, metabolism of, glucuronide formation from, in rabbits (Smith, J. N., Smithies, R. H. & Williams, R. T.) 320
Metmyoglobin complexes, mechanism of formation of (George, P. & Hanania, G. I. H.) xxxviii

Metmyoglobin complexes, pH changes accompanying formation of (George, P. & Hanania, G. I. H.) xxxvii
Milk formation and enzyme levels in mammary gland during pregnancy, lactation and mammary involution (Greenbaum, A. L. & Greenwood, F. C.) 625
Milk of cows, dietary carotene and esterification of vitamin A in (Chanda, R., Clapham, H. M. & Owen, E. C.) 453
Milk, source of antibody globulin in (Askonas, B. A., Campbell, P. N., Humphrey, J. H. & Work, T. S.) 597
Mitochondria, plant, ascorbic acid formation in (Mapson, L. W., Isherwood, F. A. & Chen, Y.-T.) 21
Monolayers, globin, effect of nucleotoxic stilbene derivatives on (Cheesman, D. F.) xlii
Monolayers of proteins and amino acid polymers, orientation of ε-amino groups in (Davies, J. T.) 509
Mucopolysaccharide, role in tendon (Jackson, D. S.) 699
Mucoproteins, urinary, isolation of (Anderson, A. J.) xxv
Muscle, skeletal, extracellular space and intracellular inorganic phosphate in (Ennor, A. H. & Rosenberg, H.) 302
Muscle, skeletal, rate of incorporation of ^{32}P into adenosine triphosphate and phosphocreatine in (Ennor, A. H. & Rosenberg, H.) 308
Myocastor coypus, coypu, bile acids of (Haslewood, G. A. D.) 581

Naphthalene, identification of 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid as metabolite of, in rabbit (Corner, E. D. S., Billett, F. S. & Young, L.) 270
Nicotinic acid in human urine, metabolites of, colorimetric method for determination of (Holman, W. I. M.) 513
Nicotinic acid levels in rabbit lens in X-ray cataract (Van Heyningen, R., Pirie, A. & Boag, J. W.) 372
Ninhydrin, use in estimating amino nitrogen in intact phospholipids (Lea, C. H. & Rhodes, D. N.) 613
p-Nitrophenol, effect on Pasteur reaction and on aerobic phosphorylation in suspensions of mammary gland (Terner, C.) 471
p-Nitrophenyl esters, reaction with chymotrypsin and insulin (Hartley, B. S. & Kilby, B. A.) 288
Nucleic acid fractions, plant, isolation for purine and pyrimidine determinations (Sherratt, H. S. A. & Thomas, A. J.) xli
Nucleic acids, chemical action of X-rays on (Scholes, G. & Weiss, J.) 65
Nucleoproteins, fission of, by strontium nitrate (Pirie, N. W.) 83
Nucleotide, removal from B_{12} vitamins (Gant, D. E., Smith, E. Lester & Parker, L. F. J.) xxxiv
Nucleotides, action of nucleotidases on (Heppel, L. A., Whitfeld, P. R. & Markham, R.) iii
Nucleotoxic stilbene derivatives, effect on globin monolayers (Cheesman, D. F.) xlii
Nutriacholic acid, identity with 3α-hydroxy-7-oxocholanic acid (Haslewood, G. A. D.) 581
Nutrient media for tissue culture (Waymouth, C.) iv

Oestrogens, colorimetric estimation of (Bauld, W. S.) 426
Oestrogens, steroid, in placental tissue, isolation and estimation of (Mitchell, F. L. & Davies, R. E.) 690
Ophidiaster ophidianus, echinoderm, carotenoids of shell of (Nicola, M. de) 555
Oral flora, metabolism of (Hartles, R. L. & Wasdell, M. R.) 353
Ox serum, electrophoretic studies of, in healthy and diseased animals (Bradish, C. J. & Brooksby, J. B.) 342; (Bradish, C. J., Henderson, W. M. & Brooksby, J. B.) 329, 335
Ox spleen, phosphoprotein phosphatase from (Sundararajan, T. A. & Sarma, P. S.) 125
Oxalate decomposing organism, citrate accumulation from glycollate by (Jayasuriya, G. C. N.) xli
Oxalic acid, effect of cyanide on formation of, by *Aspergillus niger* (Chughtai, I. D. & Walker, T. K.) 484
Pantothenate in coenzyme A formation by *Lactobacillus arabinosus* (Pierpoint, W. S. & Hughes, D. E.) 130
Pantothenic acid, phosphorylation by *Lactobacillus arabinosus* (Baddiley, J., Hughes, D. E., Mathias, A. P. & Pierpoint, W. S.) xxii
Paper electrophoresis, estimation of proteins in (Crook, E. M., Harris, H., Hassan, F. & Warren, F. L.) 434
Paper electrophoretic results, automatic scanning of (Laurence, D. J. R.) xlv
Paper ionophoresis of phosphoric esters (Neil, M. W. & Walker, D. G.) xxvii
Pasteur reaction, effect of *p*-nitrophenol on, in suspensions of mammary gland (Terner, C.) 471
Penicillium chrysogenum, metabolism of (Hockenhull, D. J. D., Herbert, M., Walker, A. D., Wilkin, G. D. & Winder, F. G.) 73
Penicillium islandicum Sopp., colouring matters of (Howard, B. H. & Raistrick, H.) 56
Pentoses, formation of D-3-phosphoglyceric acid from, by *Escherichia coli* (Bergmann, E. D., Littauer, U. Z. & Volcani, B. E.) 147
Peptide-like constituents, diffusible, of Italian rye grass (Synge, R. L. M. & Wood, J. C.) xix
Peptides, end group determination in (Evans, G. G. & Reith, W. S.) 111; (Fletcher, C. M., Lowther, A. G. & Reith, W. S.) 106; (Reith, W. S. & Waldron, N. M.) 116
Peroxidase, horse-radish, multiple components in (Jermyn, M. A. & Thomas, R.) 631
Peroxidatic activity of catalase (Laser, H.) xx
Phenols and derivatives, paper chromatography of (Myers, C. M & Smith, J. N.) 498
Phenols, metabolism in locusts (Myers, C. M. & Smith, J. N.) 498
Phenylalanine metabolism in phenylketonuria (Boscott, R. J. & Bickel, H.) i
β-Phenylethanol, metabolism of, glucuronide formation from, in rabbits (Smith, J. N., Smithies, R. H. & Williams, R. T.) 320
Phenylketonuria, phenylalanine and tyrosine metabolism in (Boscott, R. J. & Bickel, H.) i
Phosphatases, histochemical study of specificity of (Bourne, G. H.) xxi, *xxvii
Phosphate, intracellular inorganic, in skeletal muscle (Ennor, A. H. & Rosenberg, H.) 302
Phosphate, effect on cell survival in tissue cultures (Waymouth, C.) iv
Phosphate, microdetermination by paper ionophoresis (Wade, H. E. & Morgan, D. M.) 41
Phosphates, organic turnover rates of (Ennor, A. H. & Rosenberg, H.) 302, 308
Phosphatides of the latex of *Hevea brasiliensis* (Smith, R. H.) 240
Phosphocreatine, rate of incorporation of ^{32}P into, in skeletal muscle (Ennor, A. H. & Rosenberg, H.) 308
6-Phosphogluconate dehydrogenase, in lactating mammary gland (Glock, G. E. & McLean, P.) 171
6-Phosphogluconate dehydrogenase, in tissues (Glock, G. E. & McLean, P.) 171
6-Phosphogluconate dehydrogenase, in tumours (Glock, G. E. & McLean, P.) 171
D-3-Phosphoglyceric acid formation from pentoses by *Escherichia coli* (Bergmann, E. D., Littauer, U. Z. & Volcani, B. E.) 147
Phospholipids, estimation of amino nitrogen in (Lea, C. H. & Rhodes, D. N.) 613
Phosphoprotein phosphatase from ox spleen (Sundararajan, T. A. & Sarma, P. S.) 125
Phosphoprotein phosphatase, specificity of (Sundararajan, T. A. & Sarma, P. S.) 125
Phosphopyruvic acid, formation by mitochondria (Bartley, W.) 387

Phosphopyruvic acid, mechanism of formation of (Bartley, W.) 387
Phosphoric acid, dissociation constants of magnesium salts of (Clarke, H. B., Cusworth, D. C. & Datta, S. P.) xiv
Phosphoric esters, paper ionophoresis of (Neil, M. W. & Walker, D. G.) xxvii
Phosphorylation, aerobic, effect of *p*-nitrophenol on, in suspension of mammary gland (Terner, C.) 471
Phosphorylation, oxidative, in heart-muscle sarcosomes (Slater, E. C. & Holton, F. A.) 28
Phosphorylation, oxidative turnover rates of phosphate groups of flavin-adenine dinucleotide and adenosine triphosphate during (Eggleston, L. V. & Williamson, D. H.) 250
Phosphorylation with anaerobic breakdown of citrulline (Knivett, V. A.) 602
Photometry of dyed material in filter paper (Crook, E. M., Harris, H., Hassan, F. & Warren, F. L.) 434, *xxviii
Placental tissue, isolation and estimation of steroid oestrogens in (Mitchell, F. L. & Davies, R. E.) 690
Plant mitochondria, ascorbic acid formation in (Mapson, L. W., Isherwood, F. A. & Chen, Y.-T.) 21
Plant nucleic acid fractions, isolation for purine and pyrimidine determinations (Sherratt, H. S. A. & Thomas, A. J.) xli
Plasma, human, dye-binding capacity of, and determination of plasma albumin (Fildes, J. E., Laurence, D. J. R. & Rees, V. H.) xxxi
Plasma β-lipoprotein, effect on action of heparin in rabbit (Comfort, A.) ix
Plasma, radioactive components of, after administration of radioactive iodide (Brown, F. & Jackson, H.) 399
Plasmin formation from plasminogen by streptokinase (Fletcher, A. P.) 677
Plasminogen, conversion into plasmin by streptokinase (Fletcher, A. P.) 677
Polyamides, orientation of ε-amino groups in monolayers of (Davies, J. T.) 509
Polyglutamic acid, interaction with serum albumin (Hamer, D.) 610
Polyglutamic acid of *Bacillus anthracis* (Smith, H. & Zwartouw, H. T.) viii
Polymyxin, mode of action, use of fluorescent conjugates of protein in study of (Newton, B. A.) xxxii
Polysaccharide of *Bacillus anthracis* (Smith, H. & Zwartouw, H. T.) viii
Polysaccharides, protein-bound, in diabetes (Gilliland, I. C., Hanno, M. G. & Strudwick, J. I.) xxxii
Porphyrin, synthesis in haemolysates of chicken blood (Dresel, E. I. B. & Falk, J. E.) 156
Porphyrins in urine of normal men (Comfort, A. & Weatherall, M.) xix, *xxvii
Potassium and sodium, rate of turnover of, in guinea pig kidney-cortex slices (Whittam, R. & Davies, R. E.) 445
Potassium bismuth iodide, colour reactions with (Bartley, W.) 379
Procollagen, nature in rabbit skin (Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A.) 558
Progesterone, enzymic 11-β-hydroxylation of, by ox adrenocortical mitochondria (Brownie, A. C. & Grant, J. K.) xxv
Progesterone, metabolism by animal tissues *in vitro* (Taylor, W.) 463
*iso*Propylbenzene, metabolism of (Robinson, D., Smith, J. N. & Williams, R. T.) xi
Protein conversion to microbial protein in rumen of sheep (McDonald, I. W.) 120
Protein synthesis in goat followed by injection of radioactive amino acids (Askonas, B. A., Campbell, P. N. & Work, T. S.) iv
Proteins and amino acid polymers, monolayers of, orientation of ε-amino groups in (Davies, J. T.) 509
Proteins, end group determination in (Evans, G. G. & Reith, W. S.) 111; (Fletcher, C. M., Lowther, A. G. & Reith, W. S.) 106; (Reith, W. S. & Waldron, N. M.) 116
Proteins from rumen micro-organisms, nutritive value for rats (McNaught, M. L., Owen, E. C., Henry, K. M. & Kon, S. K.) 151
Proteins of rabbit skin, metabolism of (Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A.) 558
Proteins, quantitative paper electrophoresis of (Crook, E. M., Harris, H., Hassan, F. & Warren, F. L.) 434
Protoporphyrin, synthesis in haemolysates of chicken blood (Dresel, E. I. B. & Falk, J. E.) 156
Purine composition of deoxyribonucleic acids from tumours (Woodhouse, D. L.) 349
Purines, new, in B_{12} vitamins (Brown, F. B. & Smith, E. Lester) xxxiv
Pyridoxal phosphate, effect on regeneration of rhodopsin (Collins, F. D., Green, J. N. & Morton, R. A.) 493
Pyridoxine, effect on fat metabolism in rats (Desikachar, H. S. R. & McHenry, E. W.) 544
Pyrimidine composition of deoxyribonucleic acids from tumours (Woodhouse, D. L.) 349
Pyrophosphate, microdetermination by paper ionophoresis (Wade, H. E. & Morgan, D. M.) 41
Pyrophosphate, thiamine, dephosphorylation by suspensions of cerebral tissues (Pratt, O. E.) iii
Pyruvic acid 2:4-dinitrophenylhydrazone, chromatography of (Markees, S.) 703

Quinolones, orientation of biological hydroxylation in (Smith, J. N. & Williams, R. T.) 325

Rabbit skin, metabolism of collagen of (Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A.) 558
Radiation of thyroid, effect on metabolism in gland (Brown, F. & Jackson, H.) 399
Radioactivity and ultraviolet adsorption in eluate from chromatographic columns, apparatus for simultaneously measuring (Bradley, J. E. S.) xlviii
Rats, synthesis of L-ascorbic acid in (Isherwood, F. A., Chen, Y.-T. & Mapson, L. W.) 1
Retina, comminuted ox, regeneration of rhodopsin by (Collins, F. D., Green, J. N. & Morton, R. A.) 493
Retinene, effect on regeneration of rhodopsin (Collins, F. D., Green, J. N. & Morton, R. A.) 493
Rhodopsin, regeneration by comminuted ox retina (Collins, F. D., Green, J. N. & Morton, R. A.) 493
Rhodospirillum rubrum, spirilloxanthin synthesis by washed cells of (Goodwin, T. W. & Osman, H. G.) 222
Riboflavin as pigment of skins of green snakes *Philothamnus semivariegatus* and *Dispholidus typus* (Blair, J. A. & Graham, J.) 286
Riboflavin biosynthesis in *Eremothecium ashbyii* (Goodwin, T. W. & Pendlington, S.) xxiv
Riboflavin deficient mouse, liver catalase activity in (Adams, D. H.) xxvi
Ribomononucleotides, enzymic synthesis of alkyl esters of (Heppel, L. A. & Whitfeld, P. R.) ii
Ribonuclease action on ribonucleotides (Heppel, L. A. & Whitfeld, P. R.) ii; (Heppel, L. A., Whitfeld, P. R. & Markham, R.) iii
Ribonucleic acid, interaction with serum albumin (Hamer, D.) 610
Rumen, bacterial proteins of, nutritive value for rats (McNaught, M. L., Owen, E. C., Henry, K. M. & Kon, S. K.) 151
Rumen epithelium, metabolism of fatty acids in (Pennington, R. J.) 410
Rumen of sheep, protein metabolism in (McDonald, I. W.) 120
Rumen of sheep, reduction of sulphate in (Lewis, D.) 391

Rumen, protozoal proteins of, nutritive value for rats (McNaught, M. L., Owen, E. C. Henry, K. M. & Kon, S. K.) 151
Rye grass, Italian, diffusible peptide-like and glycosidic constituents of (Synge, R. L. M. & Wood, J. C.) xix

Salicylates in blood plasma, estimation of (King, E. J. & Pash, V.) xlv
Saliva, human, oxidation of sugars by (Hartles, R. L. & Wasdell, M. R.) 353
Sarcosomes, heart-muscle, oxidative phosphorylation in (Slater, E. C. & Holton, F. A.) 28
Sedimentation and diffusion of human albumins (Charlwood, P. A.) 480
Serine and glycine, interconversion of, folic acid as coenzyme for (Blakley, R. L.) xvii
Serum albumin, effect of polyacids on heat coagulation and optical rotation (Hamer, D.) 610
Serum albumins in cases of liver disease (Charlwood, P. A.) 480
Serum calcium, determination with flame spectrophotometer (MacIntyre, I.) xliii
Serum cholesterol, direct determination (MacIntyre, I. & Ralston, M.) xliii
Serum, human, arylsulphatase in (Dodgson, K. S. & Spencer, B.) xiii
Serum, ox, electrophoretic studies of, in healthy and diseased animals (Bradish, C. J. & Brooksby, J. B.) 342; (Bradish, C. J., Henderson, W. M. & Brooksby, J. B.) 329, 335
Sheep rumen, protein metabolism in (McDonald, I. W.) 120
Sheep rumen, reduction of sulphate in (Lewis, D.) 391
Shiga *see Shigella dysenteriae*
Shigella dysenteriae (Shiga), preparation and properties of the 'O' somatic antigen of (Davies, D. A. L., Morgan, W. T. J. & Mosimann, W.) 572
Silica, colorimetric estimation of, without removal of phosphate and iron (King, E. J., Pash, V. & Weedon, W.) xlvi
Silica in urine, colorimetric estimation of (King, E. J., Stacy, B. D. & Weedon, W.) xlvi
Silicic acid, inhibition of enzymes by (Kind, P. R. N., King, E. J., Pash, V., Roman, W. & Schmidt, E.) xlv
Silicotic rats, cortisone-treated, silica and collagen in the lungs and lymph nodes of (Stacy, B. D. & King, E. J.) xxxiii
Silicotic tissues, estimation of collagen in (Stacy, B. D.) xlvii
Skin of rabbits, metabolism of collagen of (Harkness, R. D., Marko, A. M., Muir, H. M. & Neuberger, A.) 558
Skyrin, colouring matter from *Penicillium islandicum* Sopp. (Howard, B. H. & Raistrick, H.) 56
Snake skins, pigments of (Blair, J. A. & Graham, J.) 286
Sodium and potassium, rate of turnover of, in guinea pig kidney-cortex slices (Whittam, R. & Davies, R. E.) 445
'O' Somatic antigen of *Shigella dysenteriae* (Shiga), preparation and properties of (Davies, D. A. L., Morgan, W. T. J. & Mosimann, W.) 572
Spectrokinetic measurements on formation of methaemoglobin-hydrogen peroxide (Dalziel, K. & O'Brien, J. R. P.) 648
Sphingomyelin, estimation in nervous tissue (Dawson, R. M. C.) 621
Spirilloxanthin synthesis by washed cells of *Rhodospirillum rubrum* (Goodwin, T. W. & Osman, H. G.) 222
Spleen cerebrosides in Gaucher's disease, lactose in (Parke, D. V.) xv
Starch and sugars, [^{14}C], simplified apparatus for photosynthesis of (Dutton, R. W.) xlviii
Steroid metabolism in suspensions of rat liver (Taylor, W.) 463
Steroid oestrogens in placental tissue, isolation and estimation of (Mitchell, F. L. & Davies, R. E.) 690
Steroids, formaldehydogenic, micro-estimation by bismuthate oxidation (Edwards, R. W. H. & Kellie, A. E.) 207
Stilbene derivatives, nucleotoxic, effect on globin monolayers (Cheesman, D. F.) xlii
Streptococcus faecalis, citrulline breakdown in cell-free extracts of (Knivett, V. A.) 602
Streptococcus faecalis, enzyme preparations from, effect of arsenate on (Knivett, V. A.) 606
Streptokinase action on plasminogen (Fletcher, A. P.) 677
Strontium nitrate, fission of tobacco mosaic virus and other nucleoproteins by (Pirie, N. W.) 83
Succinic dehydrogenase-cytochrome system, activation of (Bonner, W. D. jun.) 274
Sugar phosphates and sugars, separation by gradient elution from ion-exchange columns (Parr, C. W.) xxvii
Sugars and starch, [^{14}C], simplified apparatus for photosynthesis of (Dutton, R. W.) xlviii
Sugars and sugar phosphates, separation by gradient elution from ion-exchange columns (Parr, C. W.) xxvii
Sugars, oxidation by human saliva (Hartles, R. L. & Wasdell, M. R.) 353
Sulphatase in urine of normal and cancer patients (Boyland, E., Wallace, D. M. & Williams, D. C.) xxix
Sulphate excretion after administration of ^{35}S-labelled arylsulphuric acids to rats (Hawkins, J. B. & Young, L.) 166
Sulphate, reduction of, in rumen of sheep (Lewis, D.) 391
Sulphide, determination of (Lewis, D.) 391
Sulphides, alkyl, evolved from dog's urine by sodium hydroxide (Challenger, F., Leaver, D. & Whitaker, M. I.) ii
Sulphite, reduction by rumen micro-organisms (Lewis, D.) 391
Sulphuric acid, action on glucose and fructose, as a basis for determination of blood sugar (Mendel, B., Kemp, A. & Myers, D. K.) 639
Sulphydryl groups, relation of, with 'anti-thrombic' action of heparin (Green, J.) xxi
Synovial effusions, pathological human, flow through narrow tubes (Scott Blair, G. W., Williams, P. O., Fletcher, E. T. D. & Markham, R. L.) 504

Taurine in muscle of codling (*Gadus callarias*) (Jones, N. R.) xxii
Tendon, collagen-chondroitin sulphate linkages in (Jackson, D. S.) 699
Tendon, stabilizing linkages in (Jackson, D. S.) xv
Thermodynamics for dissociation equilibria of biologically important compounds (Ashby, J. H., Crook, E. M. & Datta, S. P.) 190, 198
Thiamine derivatives: colour reactions for, chromatography of (Bartley, W.) 379
Thiamine, determination of changes in, by a spectrophotometric method (Somogyi, J. C.) xl
Thiamine phosphates, metabolism in washed suspensions of kidney particles (Bartley, W.) 379
Thiamine pyrophosphate, dephosphorylation by suspensions of cerebral tissues (Pratt, O. E.) iii
Thiosulphate, reduction by rumen micro-organisms (Lewis, D.) 391
Thyroid activity and *S*-substituted 2-mercaptoglyoxalines (Lawson, A. & Searle, C. E.) x
Thyroxine analogue, effect on deiodination of triiodothyroxine in rats (Wilkinson, J. H. & Maclagan, N. F.) vii
Thyroxine and iodide, rapid quantitative determination of relative proportions of, after administration of radioactive iodide (Brown, F. & Jackson, H.) 399
Thyroxine, biological action of substances related to (Maclagan, N. F. & Wilkinson, J. H.) 211; (Wilkinson, J. H., Sprott, W. E., Bowden, C. H. & Maclagan, N. F.) 215

Thyroxine, detection on paper chromatograms by ceric sulphate-arsenious acid reaction (Bowden, C. H. & Maclagan, N. F.) vii
Tissue-fragments, mammalian, suspended in non-aqueous fluids, metabolism of (Rodnight, R. & McIlwain, H.) xii
Tissues, cerebral, suspensions of, dephosphorylation of thiamine pyrophosphate by (Pratt, O. E.) iii
Tissues, mammalian, with little or no added aqueous phase, metabolism of (McIlwain, H. & Rodnight, R.) xii
Tissues, sliced, apparatus for maintaining and rapidly transferring (Heald, P. J., McIlwain, H. & Nelligan, D.) xlviii
Tobacco mosaic virus, fission of, by strontium nitrate (Pirie, N. W.) 83
Tobacco mosaic virus, stability of, decreased by ultraviolet radiation (Kleczkowski, A.) 345
Toluene, expiration following administration to rabbits (Smith, J. N., Smithies, R. H. & Williams, R. T.) 317
Transpeptidation with amino acids and glutathione (Hird, F. J. R. & Springell, P. H.) 417
Tricresyl phosphates and cholinesterase (Aldridge, W. N.) 185
Triiodothyronine in rats, thyroxine analogue effect on deiodination of (Wilkinson, J. H. & Maclagan, N. F.) vii
Tryptophan metabolism in man (Dalgliesh, C. E. & Tekman, S.) 458
Tumours, purine and pyrimidine composition of deoxyribonucleic acids from (Woodhouse, D. L.) 349
Tyrosine metabolism in phenylketonuria (Boscott, R. J. & Bickel, H.) i

Ultraviolet adsorption and radioactivity in eluate from chromatographic columns, apparatus for simultaneously measuring (Bradley, J. E. S.) xlviii
Ultraviolet radiation, stability of chymotrypsin and tobacco mosaic virus decreased by (Kleczkowski, A.) 345
Urea, breakdown in tissues (Kornberg, H. L., Davies, R. E. & Wood, D. R.) 355
Urea in gastric juice (Kornberg, H. L., Davies, R. E. & Wood, D. R.) 363
Urea space in cat (Kornberg, H. L., Davies, R. E. & Wood, D. R.) 355
Urease, bacterial, in cats (Kornberg, H. L., Davies, R. E. & Wood, D. R.) 355
Urease, distribution in cats (Kornberg, H. L., Davies, R. E. & Wood, D. R.) 355, 363
Urease in cats, effect of antibacterial substances on (Kornberg, H. L., Davies, R. E. & Wood, D. R.) 355, 363
Urine, dog's, alkyl sulphides evolved from, by sodium hydroxide (Challenger, F., Leaver, D. & Whitaker, M. I.) ii
Urine, human, metabolites of nicotinic acid in, colorimetric method for determination of (Holman, W. I. M.) 513
Urine of normal men, porphyrins in (Comfort, A. & Weatherall, M.) xix, *xxvii
Ursodeoxycholic acid in coypu bile (Haslewood, G. A. D.) 581

Vesicular stomatitis, sera of cattle infected with (Bradish, C. J. & Brooksby, J. B.) 342
Viscosity, anomalous in human synovial fluids (Scott Blair, G. W., Williams, P. O., Fletcher, E. T. D. & Markham, R. L.) 504
Vitamin A, effect on regeneration of rhodopsin (Collins, F. D., Green, J. N. & Morton, R. A.) 493
Vitamin A, esterification of, in milk and blood of cows, effect of dietary carotene (Chanda, R., Clapham, H. M. & Owen, E. C.) 453
Vitamin A_2 in fishes of Nile (Abdullah, M. M., Morcos, S. R. & Salah, M. K.) 569
Vitamin B_6 deficiency, effects on fat metabolism in rats (Desikachar, H. S. R. & McHenry, E. W.) 544
Vitamin B_{12}-like factors, biosynthetic relationships between (Ford, J. E. & Holdsworth, E. S.) xxxv
Vitamins, B_{12}, new purines in (Brown, F. B. & Smith E. Lester) xxxiv
*Vitamins, B_{12}, paper electrophoresis and chromatography of substances related to (Brown, F. B., Gant, D. E., Smith, E. Lester & Parker, L. F. J.) xlix
Vitamins, B_{12}, removal of nucleotide from (Gant, D. E., Smith, E. Lester & Parker, L. F. J.) xxxiv

X-ray cataract in rabbits, changes in lens during formation of (Van Heyningen, R., Pirie, A. & Boag, J. W.) 372
X-rays, chemical action on nucleic acids (Scholes, G. & Weiss, J.) 65

Yeast protein, nutritive value for rats (McNaught, M. L., Owen, E. C., Henry, K. M. & Kon, S. K.) 151